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# Latex agglutination tests for selected Escherichia coli enzymes

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# Latex agglutination tests for selected *Escherichia coli* enzymes

by

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# GENERAL INTRODUCTION

## Statement of the Problem

The association between food and water quality and the public health of a society has been well established. To date, however, totally safe food and water supplies do not exist. Food and water supplies require continual monitoring to ensure that they are safe because many are contaminated with potentially pathogenic microorganisms and/or toxic chemicals. On the basis of sheer numbers, microbiological diseases are by far the most prominent food and waterborne threat. The cost in terms of medical expenses, lost productivity, and fatalities is enormous. Because testing all foods and water supplies for the presence of all the potentially pathogenic microorganisms is impractical, indicator organisms are used to monitor sanitary quality. Although there is debate about which organism provides the best indicator of sanitary quaiity, most laboratories conduct tests for the presence of total coliforms, fecal coliforms, and/or *Escherichia coli*. Typically, analyzing samples for the presence of these indicators is time consuming, expensive, and oftentimes lacks sensitivity and specificity. Tests for fecal coliforms and *E. coli* often require confirmation of initially presumptive reactions which takes additional assay time and labor. Furthermore, nonreactive species (e.g., anaerogenic strains of *E. coli* and *E. coli* that will not grow under the selective conditions imposed) often are not detected. Evans et al. (5) used a procedure to detect false-negative reactions (interference) in the standard most-probable-number (MPN) technique for coliform enumeration of untreated surface water and potable water supplies. Coliforms were not detected in over 80% of the samples that were MPN-negative but were verified as coliform-positive by using their verification procedure. Nevertheless, the MPN technique was superior to the standard membrane filtration (MF) technique. Jacobs et al. (9) assayed 1,483 drinking water samples collected in Vermont and New Hampshire by using MPN, MF, and presence-absence (PA) techniques. Of 336 coliform-positive samples, only 82, 64, and 88%,

respectively, were detected with the MPN, MF, and PA techniques. Thus, false-negative tests were obtained in 12 to 36% of the coliform-positive samples, depending on the assay procedure that was used. Chang et al. (2) and Clark (3) used a new 4-methylumbelliferyl-ß-D-glucuronide (MUG) test for *E. coli* that is widely used in the food industry and was recently approved by the Environmental Protection Agency for potable water testing. In the study by Chang et al. (2), a mean of 34% of *E. coli* isolated from human fecal samples yielded negative reactions when the MUG test was used. Finally, Clark et al. (3) reported that there was no significant differences in *E. coli* detected when the MF and MUG tests were used to detect *E. coli* in untreated water samples, but the MUG test was significantly inferior when used to detect *E. coli* in treated water samples. As these examples show, the techniques commonly used to test water and foods for coliforms, fecal coliforms, or *E. coli* are in need of improvement or should be supplemented with additional verification techniques.

This study was undertaken to develop rapid latex agglutination immunoassays based on the detection of enzymes specific to *E. coli* and to determine if the latex agglutination immunoassays could be used to complement traditional tests and improve the detection of *E. coli* in foods and water. It was envisioned that this assay could be used as a rapid confirmatory method for the detection of *E. coli* from presumptive-positive cultures without the need for additional labor and time intensive cultural confirmation. The original goal was to develop rapid latex agglutination assays that could be used as a one-step, 10-minute assay to confirm the presence of *E. coli* from any primary culture, enrichment broth or colony on a petri plate. In addition, the latex agglutination assays might be used to rapidly identify *E. coli* that are either glucuronidase negative with chromogenic substrates (such as those belonging to the O157:H7 serotype) or are anaerogenic, but are present in the food and water samples.

The original assay concept employed a novel method of detecting multivalent antigens by

using three separate latex particles, each of a unique color, that undergo color separation upon immunologic reactions (4, 7, 12). With the multivalent nature of the assay and the rare probability of the selected enzymes being present in species other than *E. coli*, the assay should be uniquely specific for the determination of the presence of *E. coli*, even in the presence of other organisms.

**Dissertation Format**

This dissertation is presented as five parts. The first part is a general literature review. The other four parts are presented as separate papers that may be submitted for publication in a revised form. Included at the end of each part are separate literature cited sections. An overall summary and conclusions follow the fourth part. References cited in General Introduction and General Summary and Conclusions follow the General Summary and Conclusions.

# PART 1. LITERATURE REVIEW

# INTRODUCTION

## The Food Problem

One can only imagine the problems that primitive man had with food preservation and providing safe and wholesome food to his family. Around 1837, with the beginning of bacteriology as a science and the work of Louis Pasteur, studies of food spoilage and illnesses were begun and methods to reduce or eliminate them attempted (147).

The incidence of foodborne illness in the United States is high. For a five year period (1983-1987), Centers for Disease Control (Atlanta, GA) reported over 90,000 confirmed cases of foodborne diseases (29). These cases did not represent the entire incidence of foodborne disease (146). Excluded from this information are sporadic cases of *Salmonella* infection or outbreaks involving less common pathogens such as *Bacillus cereus* and *E. coli* (29). It is estimated that only 10% of foodborne illness cases are reported (328). Estimates for the true annual incidence of food and waterborne illnesses range from 1.5 to 275 million cases (10, 11, 328). Bacterial pathogens contribute to over 60% of the foodborne illnesses confirmed in the United States annually (27, 29). New or "emerging pathogens" are receiving more attention in the literature (11, 79, 222, 328). Pathogens, such as *Listeria monocytogenes*, *Campylobacter jejuni*, *Vibrio* spp., and *E. coli* O157:H7 are now being implicated in foodborne diseases worldwide. Furthermore, the introduction of new types of food products and processing methods continually creates a variety of microbiological concerns (79, 146, 328).

## Indicator Organisms

To ensure safe food and water supplies, pathogenic or potentially hazardous microorganisms must be either absent or at "safe" levels. In this consideration, examination of every food or food product for hazardous organisms is not feasible (147). As a result,

determination of the potential for a food to harbor hazardous microorganisms is evaluated through indicator or index organisms (213).

***Criteria for indicator or index organisms***

Suitable indicator or index organisms must comply with several criteria (127, 147)y. First, the organism must be associated with the source of the pathogen. Second, the organism should be present in greater numbers than the pathogen. Third, the organism should not proliferate in the environment. Fourth, the indicator organism should be as resistant as, or more resistant than, pathogens to environmental stresses. Finally, the indicator organism should be readily recovered and identified. There are no indicator organisms that presently satisfy all these criteria.

***Indicator organisms***

Currently, several organisms are commonly recognized as indicators. These organisms form two major groups: the coliforms and the enterococci (113). In certain situations, other organisms are specifically recommended as indicators (26, 127) but these will not be discussed here.

Coliforms include several genera that are morphologically similar. Coliforms are defined as aerobic or facultative anaerobic, nonsporeforming, gram-negative rods that ferment lactose to acid and gas within 48 h at 35°C (26, 113, 133). This ambiguous definition includes predominantly *E. coli*, *Enterobacter aerogenes*, *Klebsiella* spp. and *Citrobacter* spp. Some of these are widely distributed throughout the environment, and as such have little association with public health hazards (76). Distinguishing fecal from non-fecal coliforms by the ability of fecal coliforms to produce gas from glucose when incubated at 45°C increases the usefulness of coliforms to detect the possible presence of intestinal pathogens. Currently, coliforms, fecal coliforms, and *E. coli* are used as marker organisms (113, 133).

Enterococci formerly were members of the genus *Streptococcus*. They are gram-positive

cocci that form pairs or chains, depending on the strain and cultural conditions. In general, they are catalase-positive, grow at 10° and 45°C, in 6.5% NaCl, and at pH 9.6 (126, 147). Typically, *Enterococcus* spp. are abundant in fecal material regardless of the source (244). There is some host/species association, however, absolute host specificity cannot be considered unequivocal (113, 126). A discussion of the usefulness of coliforms versus enterococci as pollution indicators is presented by Jay (147). A complete review on enterococci as indicator organisms for food and water has recently been published (155).

The choice of indicator or index organism used, however, is controversial (290). Depending on the type of food product or source of water, coliforms, fecal coliforms, *E. coli*, and enterococci each have supporters for their use (14, 44, 107, 134, 160, 275, 285).

## Rapid Methods

The adjective "rapid" means very fast. Rapid is an arbitrary term that lacks conceptual precision. It can mean different things under different circumstances to different people. In laboratory testing, rapid can take on two important meanings (158). Rapid methods can either be methods that are traditional procedures performed with more speed, or those methods that exchange or avoid steps that are time-consuming. Further demarcations of rapid methods are those with an elapsed time within the range of 4 to 12 h, and very rapid methods are those that provide results in less than an hour (106, 144, 329).

### *Reasons for rapid methods*

Rapid methods are essential elements of food and water microbiology. In most laboratories, samples and analysis parameters are increasing and time, labor, and material resources for testing are decreasing. An additional benefit is that results are obtained sooner, reducing warehousing costs for perishable foods and providing earlier recognition that a water supply might be contaminated. Overall, requirements for rapid methods are that they reduce the

time required, make an analysis easier, are less expensive (labor and materials), are better standardized, and/or may be automated (144, 145). Although a major reason for development of rapid methods is cost considerations, a primary consideration must involve specificity, sensitivity, precision, and accuracy (106). In one forecast, there was unanimous consensus that traditional methods for the enumeration of microorganisms will be replaced by automated and mechanized procedures, that rapid immunological methods will become commonplace, and that identification methods will rely more heavily on miniaturized biochemical tests or specific substrates (143). Although we are halfway through the period of this forecast, adoption of improved methods by regulating agencies and the food industry indicate that the prognostication is being met.

***Types of microbiological rapid methods***

Many methods have been published that meet some or all the criteria outlined in the previous paragraph. Some methods, including hydrophobic grid membrane filters, spiral platers, and Petrifiim "plates", are improvements on traditional methods in that accuracy is improved, a procedure is automated, or a method is made more convenient. Other methods are truly defined as rapid methods. These include methods based on bioluminescence, the direct epifluorescence filter technique (DEFT), the use of impedance and conductance, microcalorimetry, radiometry and infrared spectrophotometry, the *Limulus* lysate assay, gas chromatography, immunoassays, and DNA hybridization. It is beyond the scope of this literature review to discuss each method individually. Several excellent reviews are already available (2, 77, 91, 109, 138, 147).

# IMMUNOASSAYS

## History

Immunoassays, defined as an analytical technique based on the reaction of an antibody and antigen, were originally developed for studies of the human immune system in a medical environment (281). As the potentials of immunoassays were realized, other applications of the technique were found.

The original implementation of immunoassay technology outside studying immunology is credited to the work of Yalow and Berson in 1959 (324). Their work focused on development of an assay for insulin and in which a radiolabelled form of the hormone was used. The need for alternative methods to detect immunoassay reactions soon led to non-radioactive methods, including enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA). The use of enzyme labels for an immunoassay technique is normally attributed to Engvall and Perlmann (82) and Van Weeman and Schuurs in 1968 (307). Coons, Creech, and Jones, however, should receive more credit than they have for linking a non-radioactive label (fluorescein) to antibodies in 1941 (15). Engvall and Perlmann used the term ELISA, which alludes to one of the reactants being bound to a solid-phase (test tube, microtitration plate, etc.). For the remainder of this dissertation, the term EIA will refer to any immunoassay in which an enzyme is used to detect the presence of an antibody-antigen reaction.

## Basic Concepts and Principles of Immunoassays

To understand the potential for immunoassays in the role of a detector system, an understanding of basic concepts and principles of immunoassay is useful. Even though immunoassays are a reaction between an antibody and an antigen, many other variables exist to be exploited, or to interfere, with assay design.

### *Antibodies*

Two types of antibody preparations are available, polyclonal and monoclonal. Polyclonal antibodies are mixtures of antibodies against epitopes on an antigen. The antibody molecules will have different affinities and specificities for different epitopes. Polyclonal antibodies are relatively easy to prepare. Monoclonal antibodies are a single antibody type that results from the fusion of a plasma cell and an immortal tumor cell. Each monoclonal antibody is specific for the epitope on the antigen that the plasma cell was sensitized to produce antibody against. Preparation of monoclonal antibodies was originally described by Kohler and Milstein in 1975 (159) and has since become one of the most significant innovations in immunoassay technology.

### *Antigens*

Antigens are defined as substances that will react with a specific antibody (46). A variety of biochemical compounds, including carbohydrates, lipids, proteins, and nucleic acids, are antigenic. Smaller molecules do not normally elicit an immune reaction and production of antibodies. They can, however, act as haptens, and after linking with an immunogenic carrier molecule, can induce an antibody response (264). Throughout the remainder of this paper, the term ligand is used to represent either antibodies or antigens.

## Types of Serological Reactions

Serological reactions are those reactions that involve an antibody and antigen interaction *in vitro*. There are several useful reactions available for studying serological interactions. These reactions differ in their conditions, requirements, and sensitivities.

### *Precipitation*

Precipitation is a class of immunological reaction in which an antibody (precipitin)-antigen forms an insoluble macromolecular aggregate. If the reaction occurs in a supporting medium such as agar gel, the precipitin reaction is readily visualized and evaluated. Precipitation is the basis for

Ouchterlony and single radial (Mancini reaction) immunodiffusion reactions (48, 105, 137, 150).

***Agglutination***

Agglutination is a class of immunological reactions based on the aggregation of cells or other particulate material by antibodies. Agglutination reactions are generally more sensitive than precipitation reactions because the surface area of cells or particles is much larger than those of antigens detected in precipitin reactions (256). The agglutination reaction is the basis for many assays including hemagglutination, coagglutination, and latex agglutination.

***Radioimmunoassay***

Radioimmunoassay (RIA), as previously mentioned, heralded the inception of immunoassay technology into diagnostic work (54, 59, 189, 211, 237, 289). RIAs involve a variety of techniques in which isotope labelled reagents are used to detect either antibody or antigen. Assays are usually competitive in that radiolabelled antigen and native antigen in the sample compete for binding to an antibody (46, 289) with the amount of signal inversely generated proportional to the amount of native antigen present.

***Enzyme immunoassay***

EIAs are similar to RIAs, except that enzyme labels replace radiolabels on reagents used in EIAs. In heterogeneous EIA, activity of the label is not affected by the binding of antibody to antigen (80, 256, 289) and separation of antibody-antigen from free antibody is required. Homogeneous EIAs regulate activity of the enzyme label through binding of antigens. When antibody and antigen interact, the enzyme label is sterically activated. With no antibody-antigen interaction, the enzyme remains sterically inhibited from substrate reactions (80, 256, 289). Many variations of EIA have been developed, including direct competitive, indirect competitive, and double antibody sandwich. These different variations of EIA accommodate various analytical requirements (256, 289). Information available on EIA has grown enormously over the past

decade. Several authors have published comprehensive reviews (52, 58, 80, 256, 289).

***Affinity chromatography***

Only one author (46) has listed affinity chromatography as a separate type of immunoassay technique. This is unfortunate because affinity chromatography represents another totally different use of antibodies for analytical work. Although the basic idea of binding heterogenous molecules was originally described in 1910 (196), the technique has gained common use only within the last two decades.

Affinity chromatography involves irreversibly binding one of the components of the antibody-antigen interaction to a solid support such as agarose. The solid support with attached reactant is loaded into a column. A solution with a second reactant is passed over the column. Antibody-antigen interactions result in the retention of the reactants in the column while contaminating materials are washed away. Collection of the second reactant is effected by gently disassociating the antibody from the antigen and allowing the second reactant to pass through the column. Purification, and often concentration of the second reactant are thus achieved. Affinity chromatography has been used to detect aflatoxin from peanuts and maize (46) and is the basis for antibody purification used in this research.

## Application to Food and Water

It was mentioned previously that in a forecast concerning the future of food microbiology, immunological methods will become commonplace (143). To meet demands of rapid and sensitive detection of microorganisms and toxins, increased use of immunoassays in food microbiology is one method available. The complex matrix of food requires a method to discriminate between hazardous substances, including microbiological organisms and chemical compounds, from the non-hazardous food components. Advantage is taken of the specific selectivity of antibodies for these hazardous compounds. Applications of immunoassay

technology have been successfully applied to a wide variety of analytical problems (2, 5, 212, 223, 264, 289). There are several very good reviews on the use of immunoassays in food microbiology (5, 46, 54, 132, 223, 264, 289).

## Obstacles In Using Immunoassays

Development of any immunoassay requires specific and sensitive antibodies (4, 132) without which assay development is hampered from its inception. Initially developed for clinical diagnostics, immunoassays performed well in the examination of body fluids. But food samples are often a complex mixture of animal or vegetable tissues. Components of some foods often contain substances such as phenols and surfactants, that could interfere with immunoassays (4, 6, 132). In addition, the rigors imposed during food processing may have converted target antigen from the native form that was used to develop antibodies into another form not easily detected with those antibodies (4, 132). These obstacles, although presenting some very real challenges to development of immunoassays for food products, can, for the most part, be overcome.

## LATEX TESTS

Latex agglutination tests were originated by Singer and Plotz in 1956 (279). In hemaggiutination assays, red blood cells were the agglutination reaction indicator being used. Hemagglutination reactions date from 1922 (279) and are still used in blood transfusion work and diagnosis of many infectious diseases (220) even though they have some major disadvantages. Hemagglutination assay reagents have a stability of only a few weeks, are not well defined (they have their own surface proteins), and are not worked with easily (279).

The discovery of latex particles occurred by accident. A worker at Dow Chemical Company (Midland, Michigan) sent a sample of latex paint out for electron microscopy. Results revealed a surprising uniformity of polymeric spheres. Although it took five years to refine the process by which uniform polymeric particles were made, the particles have become very important in immunoassay technology (17). These uniform polymeric particles have become known by the generic term "latex". This is because a suspension of particles looks milky, similar to the viscous plant sap.

### Fabrication of Latex

Although uniform latex particles were not developed at Dow Chemical Company until very recently, the history of latex is centuries old, and the synthesis of polystyrene has been traced back to 1839 (128). The manufacture of styrene and other polymer resins developed because of the need for synthetic rubber during World War II (128).

The process of preparing latex is based on the polymerization of an emulsion. Two types of polymerized particles exist: homopolymers and copolymers. Homopolymers are made by polymerizing a single monomer whereas copolymers are polymerized by using more than one type of monomer. Polystyrene and polyvinyl toluene are examples of the former whereas styrene-

divinylcarboxylic acid, styrene-divinylbenzene, styrene-butadiene, and vinyltoluene-*t*-butylstyrene are examples of the latter (17, 128).

Polymerization of an emulsion to form latex requires four compounds: surfactants (e.g., lauryl sulfate or sodium dodecyl sulfate), emulsifying agent (e.g., potassium laurate), initiating agent (eg., potassium persulfate), and water. The initial process involves establishment of surfactant micelles in water. Surfactant hydrophobic-hydrocarbon tail groups aggregate at the center of the micelle and hydrophilic groups at the aqueous interface. Polymer monomers are added that migrate into the micelle's hydrophobic region. The emulsion is heated with initiating reagent which releases free radicals. The free radicals start particle nucleus formation and polymerization within the micelle. Depending on conditions of polymerization, reactants used, and amount(s) of monomers, negative surface groups are created on the polymer surface that help to stabilize the particle (18, 75, 300). Polymerization can be repeated to increase size of the particle or particles can undergo ion-exchange treatment to remove surfactant (17, 128).

By controlling polymerization reagents, the number, size, and surface characteristics of particles are changed, resulting in a extensive variety of particle sizes and types. There are four manufacturers of latex particles: Interfacial Dynamics Corporation (Portland, OR), Seradyn Inc. (Indianapolis, IN), Polysciences (Warrington, PA), and Rhône-Poulenc (Paris, France). Each manufacturer produces a broad range of particle sizes that contain a wide variety of surface groups.

### Preparation of Latex Agglutination Assay Reagents

The preparation of latex sensitized with antibodies is a multistep process similar to development of other immunoassays. While the basic procedures are similar for most immunoassays, adequate sensitization of latex is more complex than development of an EIA or RIA assay. Unlike EIA or RIA, the solid-phase becomes part of the assay, imparting a unique set of

conditions on the assay. For this reason, there are special considerations in reagent preparation.

***Cleaning latex***

It is well known that detergents decrease or inhibit the binding of proteins to polymeric surfaces (69, 103, 271, 306, 323). With current polymerization processes used by many latex manufacturers, removal of the emulsifier is necessary to obtain a well-characterized surface for protein binding. There are four adequate methods for cleaning latex particles before use: washing, dialysis, ion-exchange, and pressure cell filtration.

Washing in a buffer is the easiest method to remove emulsifiers and extraneous reactants from latex surfaces (18, 23, 271). A suspension of latex is diluted in an appropriate buffer, the latex is sedimented by centrifugation, the buffer is removed, and fresh buffer is added. The procedure is usually repeated several times. Washing is generally quick, but complete removal of all traces of emulsifier and polymerization reagents is probably not accomplished. Furthermore, complete suspension of individual particles after centrifugation often requires ultrasonication to break up clumps of particles (271).

Like washing, dialysis removes readily desorbed emulsifiers and polymerization reagents (18, 23, 68, 271). Dialysis, however, is slow and limits the molecular size of substances removed.

Ion-exchange resin cleaning of latex is reputed to be very effective in removing emulsifiers and polymerization reagents (18, 23, 271, 303). Latex is put into contact with both cationic and anionic resins, either separately or as mixed resins. The suspension is then agitated for one to two hours before being separated by filtration on a Büchner funnel. A liability in using ion-exchange resins to clean latex is the possibility of resin contamination with impurities that will adulterate the latex (271, 303).

Pressure-cell filtration, diafiltration, or serum replacement is similar to dialysis but is claimed to be much more effective than the aforementioned methods in removing emulsifiers and

polymerization reagents (3, 18, 23, 163). A filtration cell (sometimes called a stirred cell) is fitted with a porous membrane having a pore size of three-quarters to one-half the size of latex being cleaned. Ultra high purity water is fed from a reservoir into the cell under slight pressure (about 2 psi). The latex suspension is stirred for two to three days until the conductance of the filtrate attains the same level as the feed supply. Pressure-cell filtration, however, is not suitable for cleaning small research quantities of latex.

***Binding of ligand***

Attachment or sensitization of latex with ligands can be a simple process or can become quite involved, depending on the requirements of the assay. There are two methods for sensitizing latex particles with ligands: adsorption or chemical attachment. Each method has certain advantages and disadvantages.

*Adsorption* Adsorption is the simplest method of coating a ligand on latex. A suitable ligand is dissolved in a buffer solution and a suspension of latex is added and allowed to react for times ranging from a few minutes to more than a day. Latexes are generally hydrophobic and negatively charged, and they will strongly and almost irreversibly adsorb proteins by way of the hydrophobic areas in the proteins. At this point, the latex coating process may be complete and ready for use in latex agglutination assays, or additional preparation, such as blocking unreacted sites, can be further accomplished.

The diameter of a latex particle determines total surface area available for adsorbing ligands (23, 47, 69, 231, 271, 272). The amount of any proteinaceous ligand adsorbed is proportional to the diameter of the latex. The actual amount of protein adsorbed, however, is not conclusively known. Bangs calculated that approximately 2.5 $\mu$g of immunoglobulin will bind per square meter of surface area (23). A report from another commercial latex distributor, however, states that the range of binding is 4.4 to 5.8 $\mu$g of immunoglobulin per square meter (272). This

two-fold discrepancy is most likely due to the source of information that Bangs used for his calculations. The work of Cantarero et al. (47) that Bangs cited consisted of an investigation of protein adsorption to polystyrene tubes rather than particles. Thus, the data are probably not as precise as data acquired specifically from protein binding studies on latex. In addition, as previously mentioned, differences in composition and cleaning of latex influences the adsorption of proteins (231) and could affect the estimate that is obtained.

Very rarely should the entire surface of latex be covered with ligand, especially proteinaceous antigens. Because of a prozone effect (the phenomenon exhibited by some reactions in which agglutination fails to occur because of either an antibody or antigen excess; a zone outside of optimal proportions for reaction; 105) , maximum sensitivity of a latex assay requires less than saturation coverage (271).

Adsorption of proteins to latex is an incompletely understood phenomenon. Current theory is that the proteins adsorb to the latex through a combination of van der Waal's forces and hydrophobic bonds (47, 140, 306, 323). Adsorption of proteins is very rapid, taking less than an hour to reach equilibrium (231, 278).

The structure of the ligand will also influence adsorption to latex. Ligands that contain unusual chemical groups or that are highly hydrophilic are difficult to adsorb (271). Antibodies, in general, are a relatively homogenous group of proteins and adsorb readily to the surface of polystyrene particles (231). Antigens, on the other hand, are sometimes (but not always) very heterogeneous, consisting of a variety of compounds: carbohydrates, proteins, lipids, and nucleic acids. If an antigen is to be adsorbed, the chemical structure should be evaluated for presence of hydrophobic groups, without which adsorption is difficult. Covalent coupling methods may be necessary for hydrophilic antigens (271).

Different proteins have different maximal adsorption to latex (69). Whereas adsorption of

human immunoglobulin to latex was independent of pH influences, binding of human albumin and rabbit hemoglobin is dependent on pH (306). When the concentration of immunoglobulin being adsorbed was large, adsorption temperature affected binding, but for lesser concentrations of immunoglobulins or other proteins, adsorption was independent of temperature (306).

Immunoglobulins seem to be adsorbed on latex primarily by way of their hydrophobic Fc region (231, 304) whereas no specific orientation is known for other types of proteins. Conformational changes also are likely to occur as proteins are adsorbed to latex (69, 157, 231); these affect both the orientation of the proteins and the quantities bound. Desorption of adsorbed protein is generally minimized if the solution used during adsorption is not changed significantly during any subsequent manipulations of the latex (69, 231).

Latex surface structure greatly influences the amount of protein adsorbed. Sulfate-surfaced latex has a greater hydrophobicity and lower surface charge than does latex with a carboxyl surface structure. Latex with a sulfate surface therefore can adsorb more protein than latex with a carboxyl surface structure (231).

Adsorption initially increases rapidly, then plateaus following a typical bimodal adsorption isotherm (85, 231, 278). In the bimodal isotherm model, with the addition of small amounts of protein, a protein monolayer forms, latex surface charges are neutralized, and particles tend to flocculate spontaneously. A second phase occurs if more protein is added, and stability is imparted on the latex suspensions (128). The second phase adsorption has been proposed as protein-to-protein association (47, 234, 278).

Maximum adsorption of protein occurs at the isoelectric point of the protein (when its net charge is zero). This results in the protein having the most compact shape and lowest solubility (231). In addition, sensitivity of the agglutination reaction also is at its maximum at the isoelectric

point (68), although some investigators suggest that the assay pH should not be at the isoelectric point (271).

Protein adsorption to latex also is strongly influenced by ionic strength of the adsorption solution. As ionic strength of the adsorption solution is raised, adsorption theoretically increases. This is most likely because of reduction of electrostatic repulsion charges between protein and latex (231). Nonspecific agglutination reactions also are eliminated by increasing the ionic strength to 0.5 or higher (68).

*Covalent coupling* Covalent coupling of proteins to latex is used when simple adsorption is ineffective or the treated latex does not function well in an immunoassay system. Other reasons for covalent coupling include achieving a greater stability of the protein-latex binding over time and to provide specific orientation of the protein on the latex (18, 23, 60).

Several covalent coupling methods are available for attaching proteins to latex. A few common methods being used will be briefly mentioned.

Latex, modified during manufacturing to have carboxylic acid groups on the surface (carboxylate modified latex, CML), were the first useful particles used for covalent coupling (18). Generally, one can couple proteins directly to the carboxylate latex particle. As an alternative, a short hydrocarbon chain is used as a spacer between the latex and the protein being bound (18). The latter method provides a flexible linkage that allows the protein greater movement and reactivity. Direct coupling of proteins, however, is faster and easier.

Carbodiimides are groups of coupling agents that react with a protein's free amine groups and the latex's carboxylate groups. Two carbodiimides frequently used are 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimde metho-p-toluene sulfonate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (18). Coupling is either a direct reaction between protein and latex (219, 288) or through an active ester intermediate formed with the latex carboxylate groups (71,

238). The structure of the carbodiimide used, however, affects the degree of immobilization of proteins (292).

Using a spacer molecule, coupled to carboxylate latex by way of carbodiimides, reduces problems caused by steric hinderance (18, 271). Diamino alkanes, such as 1,6-diaminohexame or 1,7-diaminoheptane, added to CML particles with carbodiimides, provide an amine surface for further carbodiimide or glutaraldehyde coupling (192, 248, 250).

An investigator does not have to go through several steps to obtain reactive amine or amide group, because several manufacturers sell amino or amide modified latex. Amide groups are further reacted with hydrazine to form hydrazide-modified latex. Ligands are then coupled to the latex with a bifunctional compound, such as glutaraldehyde (18, 293).

Several other methods for covalent coupling of proteins to latex are available. Cyanogen bromide, tosyl chloride, and carbonyl imidazole are used to activate hydroxide groups (37, 149, 221, 284). Vinylbenzyl chloride and aldehyde modified particles readily react with ligand amino groups to bind ligands (271).

***Optimization***

Optimum assay conditions for latex agglutination tests are very critical for the success of the assays. Optimum conditions involve, as a minimum: the particle type, coating and blocking agents, pH, electrolyte, and presence of stabilizers (271, 318). Particle types and the effects of ligand have been previously discussed. Other considerations for achieving a suitable latex based assay will be briefly mentioned later.

Theoretical sensitivities of latex agglutination tests have been calculated (21). On the basis of a 1 $\mu$m-diameter particle, a sensitivity of 10 pg can be theoretically obtained. In actual assays, however, sensitivities have varied greatly (20). In most reports, actual sensitivities are on the magnitude of nanograms (129, 197) and only occasionally reach the theoretical limit.

Binding ligands to polymer surfaces for use in latex or other immunoassays often requires blocking of unreacted sites with molecules that have no effect on the assay (23, 81, 83, 271). Various molecules have been suggested as blocking agents (23, 240), however, bovine serum albumin and certain Tweens, such as Tween 20, are most commonly used (52, 110, 162, 204, 310).

Other compounds used to optimize agglutination reactions include high-molecular weight polymers, chaotropic agents, and halogen substituted carboxylic acids. Polyethylene glycol is probably the most commonly used polymer for increasing the degree or speed of the agglutination reaction (60, 65, 111, 130, 131, 136, 165, 193, 200, 245, 267). Polyethylene glycol is classified as an enhancing agent; a substance that increases the precipitation reaction. Through steric exclusion of water, the solubility of the antibody/antigen complex is lowered (114). Chaotropic agents (e.g., guanidinium hydrochloride, sodium thiocyanate, urea) have been used successfully in reducing nonspecific agglutination reactions (64, 67). Chaotropic agents break or weaken non-covalent bonds such as those between antibody and antigen. Nonspecific agglutinations also have been reduced by the addition of sodium trichloroacetate, a halogen-substituted carboxylic acid (283).

## Uses of Latex Particles

Improvements in methodology and continual development of new applications have ensued for latex immunoassays over the years. Initial development of latex agglutination assays, and subsequent development of methods based on latex particles, have heralded the development of many rapid methods. These rapid methods have allowed laboratories to process more samples faster, at less cost, and often with increased sensitivity than has previously been possible.

### *Changes in performance*

Since 1956, when Singer and Plotz applied latex particles to the diagnosis of rheumatoid arthritis (279), the latex immunoassay has steadily evolved. Well over 140 analytical

tests based on latex agglutination are commercially available (19, 21). Many changes in latex immunoassays have been refinements in the performance of the assay. Some manufacturers of commercial latex agglutination tests have refined the assay by simply changing the format of the test. By using capillary mixing (21, 22) instead of hand rocking, manufacturers have decreased the tedium involved in the performance of latex agglutination tests. The simple step of using dyed particles has enhanced the objective assessment of latex agglutination tests. By linking different antibodies to different colored particles, multianalyte tests are performed in one step (117, 118, 242, 257). Borrowing from their predecessors, hemagglutination and hemagglutination inhibition, latex immunoassays also have been adapted to performance in microtitration plates (21, 24, 56, 57, 128).

***Automated agglutination assays***

Development of automated instrumentation has allowed development of convenient, quantitative, and sensitive latex immunoassays. For large volume applications, such as those performed in clinical laboratories, automated methods are more desirable. Automated latex immunoassays can detect lower concentrations of reactants than are possible with manual reactions (305).

By modifying instruments originally designed for counting blood cells (33, 34, 45, 119, 175, 193) or continuous flow chemistries (35, 195), automated measurement of agglutinated particles is possible. Instruments can detect and count individual and aggregated clumps separately (318), or determine the number of nonagglutinated particles remaining after the agglutination reaction (34, 35, 45, 119, 193, 195). Several assays for clinically relevant analyses have been described (34, 35, 45, 119, 193, 195). Advantages of full automation and short reaction times do not, however, compensate for low overall sensitivity of these assays when compared with other methods, such as EIA (305).

Measurement in light absorption change by a latex suspension, either as transmitted light or as scattered light, can also be used to quantitate latex immunoassays (267). Simple spectrophotometers have been used to measure decreased absorbance in a solution of agglutinated latex particles (33, 34, 36, 172). To take advantage of newer clinical laboratory instruments that can measure changes in absorbance, several investigators have devised innovative methods for latex agglutination assays. Termed particle-enhanced turbidimetric immunoassays or PETIA, these assays have been applied to a variety of clinical measurements (65, 200, 206, 245, 301). A specially designed instrument that can measure light scattered from a suspension is called a nephelometer (20, 111, 271). Nephelometric measurements are more sensitive than simple spectrophotometric methods (optical density determinations) due to decreased background light measurements (271). Like PETIA, several clinical analyses take advantage of the speed and sensitivity of nephelometry (55, 104, 170, 206, 207). Other more advanced measurements of light scatter include quasi-elastic light scatter and angular dissymmetry (20, 21, 271, 311). Both methods simultaneously measure light scattered at more than one angle and increase the specificity of measurement of agglutination reactions (271, 311). Neither of the last methods, however, are in common use for routine latex immunoassays.

***Filter based particle assays***

There are two distinctly different approaches to using latex in combination with phase separation by filtration. Commercial applications of both are available, although the success of microparticle enzyme linked assays has overshadowed particle capture.

The principle that agglutinated particles cannot pass through a filter that nonagglutinated particles can pass through is the basis of particle capture latex immunoassay (20, 21, 22, 271). By using colored latex particles, development of a colored area on a membrane surface reveals that an agglutination reaction has occurred.

Although particle capture is not a prevalent method for detecting latex immunoassays, a derivative, microparticle enzyme linked assays (MEIA) (20, 21, 22, 164, 271) have captured a large market in clinical laboratories. Similar to particle capture assays, MEIA starts with antigen or antibody bound latex particles and a filtering device. Unlike particle capture assays, MEIA uses an enzyme/substrate method to detect agglutination. Unlike membrane dot blots, the use of latex particles increases the surface area of the reaction. That, in turn, increases the ease of use and reduces assay time (8, 39, 190, 191, 302, 317).

***Latex chromatography***

Within the past few years, another novel use of latex particles has been successfully commercialized. Based on the migration of dyed latex along a filter strip, this method employs a sandwich antibody capture to form a detectable signal (21, 22, 38). Antibody coated particles are spotted on a nitrocellulose strip. When the strip is dipped into a solution, the particles migrate with the solution up the strip by capillary action. If the solution contains an appropriate antigen, the particles will become bound by a second immobilized antibody. The antigen becomes the link that holds the labeled antibody to the immobilized antibody. If the solution does not contain the appropriate antigen, the particles continue to migrate past the second capture antibody and no spot will develop.

***Magnetic particles***

Magnetic particles have become widely used for immunoassays because of their ability to be readily removed from reactions and washed free of interfering agents. Although the particles are termed "magnetic", the particles are not themselves magnetic but respond to a magnetic field (21). Antibody coated magnetic particles have been used to "capture" a host of substances, including human cells and proteins (262), bacteria (96, 176, 178, 229, 298, 309), viruses (121, 201, 226), and parasite proteins (115). After capture, antigens are detected with enzyme immunoassay

(115, 176, 262) or other methods. When capturing bacteria, polymerase chain reaction (PCR) analysis (214, 316) or growth in selective media (96, 178, 229, 309) can be used to identify the captured organisms. Lim et al. (171, 173, 174) used a second latex particle coated with antibody as the indicator system. Antigen, if present, becomes sandwiched between the magnetic particle and the indicator particle, allowing removal of the magnetic and indicator particles from the preparation. If no antigen is present, removal of the magnetic particles from the preparation leaves the indicator particles in the preparation.

***Radioimmunoassay***

To reduce incubation times and improve bound/unbound separation, latex particles are used in radioimmunoassays (21, 261). Commercial applications of magnetic particles in radioimmunoassays are available (22).

***Nucleic acid assays***

With the use of nucleic acid techniques increasing, application of particles to nucleic acid hybridizations and amplifications are natural extensions of immunoassays (21). Nucleic acids are directly attached to particles (321) or are captured through intermediates such as biotin (21, 214). Latex particles also have been used to capture PCR-amplified products (308).

***High density particles***

High density latex particles (approximately 1.5 $g/cm^3$) have been used in both a reversed passive latex agglutination test and a sandwich assay. Using high density particles in a reversed passive latex agglutination test decreased the assay time from 16 h to 3 h (99). A high density particle can be combined with an indicator particle in a sandwich assay, similar to two-particle magnetic immunoassays. This allows detection of analyte after centrifugation of the high density particle; no further processing steps are needed (167).

***Fluorescent microparticles***

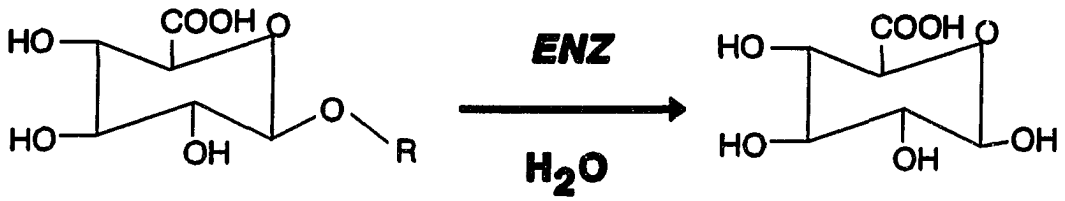
Latex particles, into which fluorescent dyes have been incorporated, have a multitude of novel uses. Fluorescent particles are being used to label cell surface antigens for microscopic work (41, 205, 251), determine antibodies or cell types in flow cytometry (75, 198, 199, 291), undergo phagocytosis for virus research (41), trace neuron pathways (41), and signal different MEIA applications (41, 164, 236).

## THE ENZYMES

### Glucuronidase

*Properties*

Glucuronidase is an acid hydrolase that hydrolyses ß-D-glucuronides (testosterone, oestriol, pregnanediol, and others) to D-glucuronic acid (92, 168, 169). It is a tetrameric




glycoprotein present in tissue lysosomes, endoplasmic reticulum (169, 235, 239) and bacteria (42). The enzyme contains a high concentration of polar amino acids and the sugars include galactose, mannose, glucose, and hexosamine (235).

Mouse glucuronidase is described as having a subunit molecular weight of 69,000 to 82,000 and whole unit molecular weight of 280,000 to 300,000 (102, 235). In comparison, bacterial glucuronidase from *E. coli* has a monomeric molecular weight of 68,200 (148). The genes encoding bacterial enzyme activity were isolated and characterized in 1986 by Jefferson et al. (148). The operon maps at minute 36 on the *E. coli* chromosome and consists of four separate genes (225). Regulatory functions are controlled by a 600 bp gene, *uid*R. Enzyme activity is encoded by a 1806 bp gene, *uid*A. A glucuronide-specific permease is encoded by a 1371 bp gene, *uid*B. A fourth gene of 1125 bp, *uid*C, has an unknown function. Within some groups the genotypic designation of the operon has been changed from *uid* to *gus* (319). The change of the operon designation is based on glucuronidase work done in the mouse model and subsequent revision of mouse genetic nomenclature (cited in 235)

Glucuronidase is inducible by glucuronide and fructuronic acid substrates (224) but the efficiency of induction varies among bacterial strains (125). A regulatory gene encodes an operon

repressor and loss of the regulatory gene leads to constitutive glucuronidase activity (319). Besides repressor activity, the glucuronidase operon appears to have a CAP binding site that prevents transcription in the presence of carbohydrates (265, 319). Another source of glucuronidase repression is under the control of another operon's regulatory gene. Repression of the *uxu* operon (that encodes for further metabolism of glucuronic acid) represses glucuronidase transcription (225).

The pH optimum of the glucuronidase enzyme from *E. coli* is between 5.0 and 7.5 (148); however, the pH optimum may vary, depending on the substrate (125). Glucuronidase resists thermal inactivation at 50°C (148). Heavy metals (13, 72, 169) and ascorbic, citric, and some other organic acids (239) inhibit glucuronidase activity. Chelating agents can increase or inhibit glucuronidase activity (13, 125).

***Methods of detection***

Several fluorometric and nonfluorometric substrates are available to detect glucuronidase activity (218, 297). Most of the substrates contain D-glycopyranosiduronic acid attached by way of a glycosidic linkage to a hydroxyl group of a detectable molecule (218). Hydrolysis of the hydroxyl group results in cleavage of the glycosidic bond and release of the detector molecule. The glucuronidase from *E. coli* has a specificity for ß-conjugated glucuronides and will not cleave other glycosides (218).

There are several methods for detecting glucuronidase activity on the basis of histochemical demonstration of the enzyme. These involve hydrolysis of 8-hydroxyquinoline glucuronide in the presence of acid-ferrocyanide reagent, 1- or 2-naphthyl glucuronide or naphthol AS ß-D-glucuronide with post coupling to a diazonium salt, or indoxyl glucuronides (218, 239). 5-bromo-4-chloro-3-indolyi ß-D-glucuronide (BCIG), although expensive, is probably the best substitute for colorimetric assays and is the recommended histochemical method (218). The

colorless substrate is first hydrolyzed releasing an indoxyl group. The indoxyl group is then dimerized and oxidized to a water-insoluble indigo dye. BCIG has been used to identify glucuronidase-positive bacteria. Watkins et al. (313) added BCIG to both lauryl tryptose broth and mTEC agar as a direct indicator of glucuronidase activity. Frampton et al. (95) and Restaino et al. (252) incorporated BCIG into peptone-tergitol agar for direct glucuronidase detection. All three media were successfully used to identify *E. coli* from food sources.

Another colorimetric substrate is p-nitrophenyl ß-D-glucuronide (PNPGlu) (218). Cleavage of the bond between the phenol group and glucuronic acid liberates 4-N-nitrophenol that is measured spectrophotometrically at 402-410 nm. 4-nitrophenol is water soluble and less sensitive than other substrates, limiting its usefulness for some purposes. Adams et al. (1) used this substrate in a tryptone broth to study environmental water samples. They found that using a threshold optical density cutoff, detection time was inversely linear to the concentration of *E. coli* present.

The most widely used substrate for detection of glucuronidase activity is 4-methylumbelliferyl ß-D-glucuronic acid (MUG). Hydrolytic cleavage yields a fluorescent 7-hydroxy-4-methyl coumarin that when excited at 363 nm, fluoresces at 447 nm. This substrate is two to thirty times more sensitive than PNPGlu and certain other substrates (218). 7-hydroxy-4-methyl coumarin exists in both a phenolic and phenoxide form, depending on the pH (50). At a higher pH (greater than pH 8.2), the more fluorogenic phenoxide form exists, whereas at a lower pH (less than pH 8.2), the non-fluorogenic phenolic form exists. The excitement of MUG can readily be achieved with long-wavelength ultraviolet light (365 nm) with visual detection of the blue fluorescence.

Other fluorogenic substrates for glucuronidase are resorufin ß-D-glucronic acid and 4-trifluoromethylumbelliferyl ß-D-glucuronic acid (TFMUG) (218). Cleavage of the substrate yields

resorufin which emits a fluorescence at 584 nm when excited at 571 nm. Resorufin, unlike MUG, has maximal fluorescence at neutral pH and is stable except in the presence of reducing agents. Like resorufin ß-D-glucronic acid, TFMUG ($pK_a$ 7.3) has increased fluorescence at the more neutral pH values. TFMUG has an excitement wavelength of 393 nm with an emission of 502 nm. Further information on glucuronidase substrates is found in reviews by Feng (88) and Manafi et al. (184).

Several investigators have applied nucleic acid-based methods to detect the presence of the genes encoding for glucuronidase activity. Cleuziat and Robert-Baudouy (53) used three different gene probes based on *uid*R or *uid*A gene fragments and polymerase chain reaction (PCR) DNA amplification to test the usefulness for detecting *E. coli* from collection strains. They found that 78% of *Escherichia* and *Shigella* strains hybridized with probes. Of those strains, 43% were phenotypically negative. Two strains of non-*Escherichia/Shigella* spp. were phenotypically glucuronidase-positive but gene probe-negative.

Bej and co-workers (30, 31, 32) used PCR and DNA probes to extensively study *E. coli* and coliforms in water. Although their original assays did not target glucuronidase genes, they later started using glucuronidase genes as targets. Their original assays used PCR amplification of gene fragments from *lac*Z and *lam*B (32). Their detection limits were one to five viable *E. coli* cells per 100 ml of water; however, the *lac*Z and *lam*B gene fragments were not specific for *E. coli*. When they switched to *lac*Z, *uid*R and *uid*A gene targets, the assay became specific for *E. coli* and *Shigella* species, including four MUG-negative *E. coli* strains (30). Estimated sensitivity was calculated to be one to two bacterial cells when the *uid*R gene was used. In a comparison between MUG-containing media and PCR detection using *uid*A gene probes, there was, or nearly was, a statistically significant difference in the detection when used to monitor water quality (31).

Green et al. (112) used a gene probe based on an *E. coli* K-12 glucuronidase gene to detect fecal contamination of water. A 10 h non-selective enrichment period after filter membrane

isolation was optimum. Only 90% of *E. coli* strains isolated from environmental sources were phenotypically glucuronidase-positive, but all were gene probe-positive. Other than *E. coli*, only *Shigella* spp. were phenotypically and gene probe-positive for glucuronidase. Feng et al. (90) studied phenotypically glucuronidase-negative *E. coli* with a synthetic gene probe for part of the glucuronidase gene. Ninety-seven percent of *E. coli* were gene probe-positive, despite the phenotypical expression. Of particular importance in this study was the inclusion of *E. coli* O157:H7 that are typically glucuronidase-negative with MUG (74) but are 100% positive by gene probe for the glucuronidase gene.

***Bacterial identification***

In 1949 Buehler et al. (42) demonstrated that certain strains of *E. coli* and two species of *Corynebacterium* produced ß-glucuronidase. Since that time, glucuronidase activity has been used to detect *E. coli* as an indicator organism in many food and water methods. Glucuronidase activity is a characteristic limited primarily (among the gram-negative bacteria) to *E. coli* and about half the *Shigella* strains (108, 122, 153, 243, 273, 299, 319). In Killan and Bülow's original report (153) on using glucuronidase for identification of *E. coli*, they reported that 97% of *Escherichia* strains, in which they included *Shigella* spp., possessed glucuronidase activity. Chang et al. (49), however, reported that 34% of their *E. coli* isolates were glucuronidase-negative. Suggestions about the differences in results obtained by Chang et al. (49) and those of other investigators were presented, but no evidence to support any of them was given. A further study was undertaken in which the *E. coli* reference collection (ECOR) was used (177). The standardized collection contains a representative population of *E. coli* strains and is available from the American Type Culture Collection (Rockville, MD) as well as other sources. Results of this study demonstrated that many more fecal *E. coli* are glucuronidase-negative than *E. coli* from other sources, and the results were consistent with their previous report (49). In contrast, urinary isolates of *E. coli* were

100% positive for glucuronidase activity (177). In a later study (254), involving human, bovine, and equine fecal *E. coli*, 96% of the isolates were glucuronidase-positive. These studies suggested that the exact percentage of *E. coli* that have glucuronidase activity is dependent on the source and test methodology.

A unique serotype of *E. coli*, O157:H7, is described as most often negative for glucuronidase activity (73, 74, 161, 249, 270). This strain is the causative agent of hemolytic uremic syndrome and hemorrhagic colitis, and it is a significant public health threat. The genus *Escherichia* also includes *E. adecarboxylata*, *E. blattae*, *E. fergusonii*, *E. hermannii*, and *E. vulneris*; these are generally negative for glucuronidase activity (253). One strain of *E. vulneris*, however, has been described as being positive (53).

Other than *E. coli* and *Shigella* spp., the overall occurrence of glucuronidase producing organisms within the family *Enterobacteriaceae* is low (125). Some investigators have described strains of *Hafnia alvei* that are glucuronidase-positive (62, 274) although others have not found any glucuronidase positive *Hafnia* spp. (153, 255). Le Minor et al. (166) characterized 30% of the more than 4000 strains of *Salmonella* that they studied as having glucuronidase activity. Sharpe et al. (274) reported that 20% of 566 *Salmonella* strains tested were positive for glucuronidase activity. Kämpfer et al. (151) described five of nine *S. arizonae* that were glucuronidase-positive but none of *S. typhi* (14 strains) or *S. enteritidis* (6 strains) tested were positive. Feng and Hartman (89) and Trepeta and Edberg (299) stated that 17 and 15% of the *Salmonella* strains that they examined were glucuronidase-positive. But, Kilian and Bülow (153) reported that none of their ten strains of *Salmonella* were positive. The last four studies, however, did not involve as many strains as were studied by Le Minor et al. (166) or Sharpe et al. (274). As pointed out by Feng and Hartman (89) and Damaré et al. (62), although glucuronidase-positive *Salmonella* limit specificity of using glucuronidase solely for identification of *E. coli*, it does not hinder the intended public health

intention of MUG use for rapid identification of potential contamination. In some reports, some strains of *Citrobacter* and *Enterobacter* have been described as producing glucuronidase (125, 313), but the overall occurrence of these organisms producing glucuronidase is less than 10% of those studied. In one report an *Enterobacter cloacae* was phenotypically, but not genotypically, glucuronidase-positive (53). In this report the authors question the phenotype, hence the identity, of the organism. *Yersinia kristensenii* also has been described as having strains that are glucuronidase-positive (four of nine strains) (151). A complete review of the distribution of glucuronidase in selected genera and species has been published (125) as well as a survey of glucuronidase activity within the family *Enterobacteriaceae* (151) and for use in food, water, and clinical samples (94).

Other gram-negative non-coliform bacteria have been reported to have glucuronidase activity. Petzel and Hartman (243) reported some strains of *Flavobacterium* spp. produced glucuronidase. This problem was seen in cultures from frozen foods that were allowed to grow on a medium selective for gram-negative bacteria when the plates were incubated for over 20 h. *Aeromonas* spp., a common waterborne organism, has demonstrated a few glucuronidase-positive strains (125, 266).

Some strains of *Staphylococcus*, *Micrococcus* and *Streptococcus* have glucuronidase activity. Robinson and Moberg (202, 258) isolated glucuronidase-positive staphylococci and streptococci from lauryl sulfate tryptose broth supplemented with MUG. In another study, staphylococci and a *Micrococcus* sp. isolated from peptone tergitol glucuronide agar produced glucuronidase (62). In addition, a single species of *Bifidobacterium* is described as having glucuronidase activity (260). Other gram-positive organisms described as having glucuronidase activity include *Clostridium* spp. (263) and *Corynebacterium* spp. (61). To avoid problems with using glucuronidase activity for identification of *E. coli*, the presence of gram-positive organisms

can readily be discriminated by selective culture (202).

Other non-coliform glucuronidase producing bacteria include a gliding bacterium (thought to be *Myxobacteria*), an organism similar to either a *Micrococcus* or *Clavibacter*, an unidentified bacterium (similar to either staphylococci or brevibacteria), and a *Curtobacteria* spp. (296, 319). The significance of these reactions has not been addressed in using glucuronidase activity outside of plant genetics, the source of this information. Further assessment of these organisms and the likelihood of their presence in non-contaminated food and water needs addressing.

Glucuronidase substrates have been used in a variety of media, under a variety of conditions, and for a variety of samples. Liquid media such as lauryl tryptose broth (9, 49, 89, 177, 202, 241, 255, 258, 280, 313), lactose broth (7), m-Endo broth (7, 89, 98), m-FC broth (98), EC broth (9, 156, 255, 273), MacConkey broth (63), MacConkey-purple broth (16), brilliant green 2% bile broth (63), and ß-glucuronidase-tryptone broth (1) have all been described as having certain advantages and disadvantages, depending on the application. Solid media such as violet red bile agar (7, 89, 315), MacConkey (299, 315), peptone tergitol agar (62, 95, 98, 227, 252), LES Endo agar (194), tryptone bile agar (268, 274), lactose-monensin-MUG agar (98), tergitol-monensin-MUG agar (98), MUG-7 agar (265), buffered MUG agar (84), m-LGA (266), EMX ID-agar (185), and m-FC (98, 194) were used with similar results as the broth media.

Lauryl tryptose broth and buffered MUG agar have been adopted by the Association of Official Analytical Chemists for official use in testing (84, 203). Two commercially available rapid methods for detecting coliforms and *E. coli* are available and are subject to controversy (78, 232). In the study by Clark et al. (51), these commercial media had respective failure rates of only 12% and 19% for untreated water but a failure rate of 61% and 81% for treated water (when compared to membrane filtration for detection of *E. coli*). When compared to standard membrane filtration for total coliforms, commercial methods fared better with greater than 94.8% agreement (233). Further

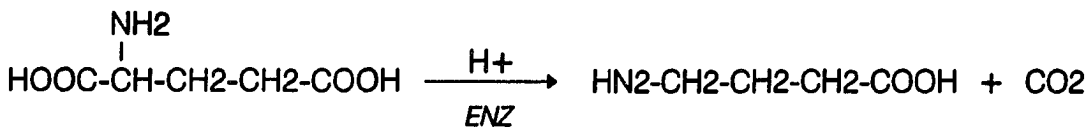
information on the use of glucuronidase activity in differentiating *E. coli* in foods and water was reviewed by Hartman (125).

## Glutamate Decarboxylase

Although previous investigators described the production of amino acids, including γ-aminobutyric acid from glutamate by bacteria, Gale (100), in 1940, was the first to describe strains of *Bacterium coli* (*E. coli*) that have glutamate decarboxylase. In further studies (101), he proposed that decarboxylase activity is used by bacteria to maintain physiological pH under acidic conditions.

### *Properties*

Glutamate decarboxylase is a pyridoxal-phosphate containing enzyme. The enzyme is a hexameric molecule with a total molecular weight of approximately 310,000 and a subunit molecular weight of approximately 52,000 (282, 286). The subunits are composed of nearly identical proteins (286). The optimal pH for enzyme activity is 3.8 (276, 286). Glutamate decarboxylase is responsible for the decarboxylation of glutamic acid; carbon dioxide and γ-aminobutyrate are produced (93). The enzyme is inducible and is produced in large amounts by




*E. coli* when grown in a medium containing L-glutamate (93). Glutamate decarboxylase is specific for L-glutamate and will not decarboxylate D-glutamate (101). Uniquely, glutamate decarboxylase is less stable at low temperatures than at room temperature when in neutral preparations (277). A study using electron microscopy (294) contributed to the knowledge that the enzyme has a hexameric structure consistent with its subunit composition, and the study confirmed that low temperature instability was structurally related.

Lupo and Halpern (179, 181) used immunological methods to study glutamate

decarboxylase. They concluded that there were no immunological differences among glutamate decarboxylases from different strains of *E. coli* with different enzyme activities. Their later work, with anti-glutamate decarboxylase and mating experiments, lead to the conclusion that glutamate decarboxylase is under control of a regulatory gene that is responsible for differences in activity among bacterial strains (180).

Smith et al. (282) recently recounted that glutamate decarboxylase activity is under the control of two distinct genes. The two genes, *gltA* and *gltB*, both code for very similar, but not identical, proteins. This information contradicts the resuits of eariier works (180, 187, 188) on the location of *gadS* and a regulatory gene, *gadR*, determined by genetic analysis. The amino acid sequence of *E. coli* glutamate decarboxylase has been determined (186) and has some similarity to those of mammalian glutamate decarboxylases. Divalent metal cations, including $Zn^{2+}$, $Hg^{2+}$, and $Cu^{2+}$, are inhibitors of enzyme activity (326). In addition, conformational analogues, such as 4,5-dihydroxyisophthalic acid, chelidonic acid, and 4-bromoisophthalic acid are potent inhibitors (326).

***Methods of detection***

Paper chromatography was one of the original methods employed to detect glutamate decarboxylase. King and Fletcher (154) and Proom and Woiwod (246, 247) used paper chromatography to detect the presence of an end product, γ-aminobutyric acid, in culture fluids. The two research groups used different solvent systems, however, both used ninhydrin as the developing agent. Proom and Woiwod (247) later studied the production of glutamate decarboxylase by members of the family *Enterobacteriaceae* and genus *Aerobacter* as a method to classify species.

Møiler (215) used manometric measurements of carbon dioxide evolution to study *Enterobacteriaceae* decarboxylases, including glutamate decarboxylase. Moran and Witter (209)

developed an automated test for detecting glutamate decarboxylase activity. Using a Technicon AutoAnalyzer, they were able to liberate carbon dioxide into a phenolphthalein indicator solution and measure the decrease of absorbance as indicator of the presence of *E. coli* in the original sample. The detection level was determined to be 50,000 cells/ml of sample and was described as being useful in milk (209, 320) and water samples (208). Measurement using a carbon dioxide sensitive electrode also has been used (327) but suffers from slow response time to the liberated carbon dioxide.

Møller (217) later developed a liquid medium in which decarboxylase activity from microorganisms could be detected. This method, based on decarboxylation of amino acids, results in an alkaline pH shift of the medium. This method, and its modifications (86), are the primary method for detecting the decarboxylation of lysine and ornithine. The detection of glutamate decarboxylase activity, however, requires careful and exact manipulation of the pH of detection media and the method is too exacting for routine use. A rapid resting-cell method of detecting glutamate decarboxylase activity has been developed (314), but is not in widespread use. In this method, a heavy suspension of toluene-treated bacteria in 0.1% sodium glutamate with brom cresol green as an indicator is used. This method normally required only three to four hours of incubation at 37°C before results were interpretable, but overnight incubation was necessary for some organisms. Schubert et al. (269) later used a similar method in their study of glutamate decarboxylase activity. Instead of using a glutamic acid solution, they impregnated filter paper disks with glutamic acid. Toluene-treated cells were sedimented by centrifugation and suspended in an acid-water solution. After 20 min, brom-cresol green indicator solution was added and results were recorded the further incubation.

Many other methods to study glutamate decarboxylase have been used. Radiolabelled glutamate has been used to study enzyme activity (124). Using an acid-base indicator, Rosenberg

et al. (259) developed a spectrophotometric method. Although their method was more rapid and convenient than manometric methods, it was less sensitive. None of these glutamate decarboxylase procedures are in common use for routine bacterial classification.

Several agar-based methods for detecting decarboxylase activity have been described in the literature. Burman and Östensson (43) described a medium composed of decarboxylase broth base with added agar to detect lysine, ornithine, and arginine decarboxylases. Maccani (182) developed a decarboxylase test agar for the determination of lysine and ornithine decarboxylation without anaerobic incubation. Yamani and Untermann (325) described a decarboxylation medium that has been used for histidine, lysine, ornithine, and arginine decarboxylation determinations. Reacting amines from decarboxylation reactions with nitrosallcylaldehydes formed colored nickel complexes in another method (142). The latter method has been used for lysine and ornithine decarboxylase determinations. No reference, however, was made to using any of these methods for glutamate decarboxylase.

A method for finding glutamate decarboxylase from brain tissue is based on the detection of NADPH (228). The reaction is based on the fact that glutamate decarboxylase produces γ-aminobutyric acid which then combines with ketoglutarate and continues through two steps to succinate and NADPH. Although no bacterial application has been described, it is doubtful that this method would be practical for bacterial classification.

***Bacterial identification***

Proom and Woiwod (247) originally observed that glutamate decarboxylase production was limited to *Bacterium coli* (reclassified as *E. coli*) and *Shigella* spp. among the *Enterobacteriaceae*. In later studies by Møller (217), 99% of *E. coli*, 83 to 89% of *Shigella* spp., 69% to 100% of *Proteus* spp., 100% of *Providencia*, and only a few *Hafnia* spp. were described as having glutamate decarboxylase activity. Other *Enterobacteriaceae* were negative. Although, in an earlier report (216) a few rare *Enterobacter* and *Klebsiella* spp. were described as positive for

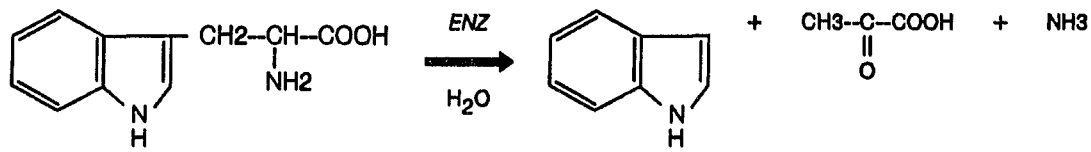
glutamate decarboxylase activity, the method used was paper chromatography rather then a metabolic method. Using the rapid method of Wauters and Cornelis (314), Freier et al. (97) established that 96% of *E. coli*, 71% of *Proteus* spp. (*P. vulgaris* - 100% negative), 100% of *Shigella* spp., and 18% of *Providencia* spp. (*P. alcalifaciens* - 100% positive) were positive for glutamate decarboxylase activity whereas other *Enterobacteriaceae* species were negative. In addition, several species of anaerobic bacteria, including strains of *Bacteroides* spp., *Clostridium* spp., *Fusobacterium* spp., *Eubacterium limosum*, *Propionibacterium acnes*, and *Peptostreptococcus* sp., also had glutamate decarboxylase activity (25, 97). When testing for enteric contamination, these latter organisms are selectively excluded in glutamate decarboxylase testing. Glutamate decarboxylase also is produced by some strains of *Listeria monocytogenes* and *Mycobacterium leprae* (cited in 28), but this should not limit the usefulness of glutamate decarboxylase for *E. coli* identification.

## Tryptophanase

Distinguishing between two organisms within the *Enterobacteriaceae* by using indole production was originally described by Kitasato in 1889 (cited in 141). Indole production is now one of the most commonly used tests to differentiate *Enterobacteriaceae* and is used in most commercial identification systems.

### *Properties of the enzyme*

*E. coli* tryptophanase also is a pyridoxal-phosphate containing enzyme (210, 322). The apoenzyme is a tetrameric molecule with a total molecular weight of approximately 209,000 to 220,000 and a subunit molecular weight of approximately 55,000 (210). Each tryptophanase subunit contains a combining site for pyridoxal-phosphate (210). Tryptophanase catalyses a reversible deamination of the L-tryptophane side chain resulting in the formation of indole, pyruvate, and ammonia (123, 322). The reaction requires water, the coenzyme pyridoxal




phosphate, and monovalent cations such as $K^+$ and $NH_4^+$ for activity (152, 210). In addition to ß-elimination, tryptophanase can catalyze *è*-elimination, ß-replacement, and *è*-hydrogen exchange reactions with amino acids (152). The optimal pH for tryptophanase activity is 7.5 (116).

Tryptophanase is inducible by tryptophan and its analogs (66), and peptones used in media to detect indole must contain enough tryptophan to induce enzyme production (295). Tryptophanase synthesis is repressed by growth in the presence of carbohydrates such as glucose, lactose, xylose, or maltose (40).

***Methods of detection***

Detection of indole production, although a good means of differentiating among bacteria, does have some inherent problems (141). Two different reagents are in common use to determine indole production: Ehrlich's reagent and Kovac's reagent. Both reagents consist, in part, of *p*-dimethylaminobenzaldehyde. Indole formed from the deamination of L-tryptophan combines with the aldehyde in a multistep reaction to form a red colored quinoidal compound (183). In routine use, these reagents are either added to broth cultures or broth cultures or colonies are applied to filter paper impregnated with the reagents. A more recent reagent that has been applied to testing indole production is *p*-dimethylaminocinnamaldehyde (124). The latter reagent gives more intensely colored reactions, increasing test sensitivity by a factor of ten. Inasmuch as use of this reagent detects cultures that produce small quantities of indole, some cultures formerly deemed indole-negative when other reagents were used, would be considered indole-positive when the new, more sensitive reagent is used.

In what can arguably be called the origin of rapid, miniaturized biochemical tests for bacteria, Arnold and Weaver investigated a microtechnique for indole detection (12). Using 1 ml quantities of media, a heavy inoculum, and incubation at 37°C for up to six hours, Arnold and Weaver (12) found that results were identical to four-day methods. Their results later lead to development of rapid, miniaturized fermentation tests for bacterial identification (120). In an attempt to achieve even more rapid test for indole production, Vracko and Sherris (312) investigated using *p*-dimethylaminobenzaldehyde as an indicator for a rapid spot test. By applying bacteria to filter paper prewet with the reagent, results were comparable to the results of the more venerable Kovac's test and were available within seconds.

Other methods for determining tryptophanase activity also are available. Tryptophanase activity is determined by monitoring the reduction of NADH as lactate dehydrogenase converts an end product, pyruvate, to lactic acid (210, 287). Radiolabelled tryptophan has been used as a substrate for tryptophanase activity in a method used to determine the content of pyridoxal phosphate (230). A direct spectrophotometric assay of tryptophanase on the basis of a chromogenic substrate, S-*o*-nitrophenyl-L-cysteine, also has been described (287). In this method, tryptophanase causes an è,ß-elimination, forming *o*-nitrothiophenolate; the reaction is monitored at 370 nm. This method is not as sensitive as the lactate method nor as easy to perform as the *p*-dimethylaminobenzaldehyde or *p*-dimethylaminocinnamaldehyde reagent methods.

***Bacterial identification***

Tryptophanase activity has been described in many fecally associated bacteria (66). These include *E. coli*, *Citrobacter* spp., *Edwardsiella* spp., *Klebsiella* spp., *Kluyvera* spp., *Leclercia adecarboxylata*, *Morganella* spp., *Providencia* spp., *Bacillus* (*Hafnia*) *alvei*, *Proteus vulgaris*, *Aeromonas liquefaciens*, *Bacteroides* spp., *Corynebacterium acnes*, *Micrococcus aerogenes*, *Photobacterium harveyi*, *Pasteurella multocida*, and some *Yersinia* spp. and *Serratia odorifera*

spp. (66, 87, 135). Although tryptophanase activity is not limited to *E. coli*, the incorporation of indole detection has evolved as a useful adjunct test to identify *E. coli* in samples of shellfish, urine, and water (70, 139, 268).

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# PART 2. PRODUCTION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES TO SELECTED *E. coli* ENZYMES

## INTRODUCTION

The development of a diagnostic assay based on immunological reagents requires the availability of specific and sensitive antibody preparations. Kaspar (31) and Holt (21) used commercial *Escherichia coli* enzyme preparations to produce polyclonal anti-enzyme antibodies, some of which seemed to have the specificities and sensitivities needed for coagglutination and enzyme-capture tests. Therefore, to obtain antisera for use in developing rapid latex-agglutination assays, rabbits were immunized by using the purest commercial enzyme preparations available of *E. coli* glucuronidase, glutamate decarboxylase, and tryptophanase. The results of initial latex agglutination tests using these antibody preparations suggested, however, that characterization of the antibody preparations was necessary because cross-reaction were observed. Studies on antibody purity, cross-reactivity, and functionality were determined by using polyacrylamide gel electrophoresis, Ouchterlony immunodiffusion, and enzyme immunoassay techniques. Later, because Ouchterlony immunodiffusion assays yielded ambiguous results, Western blots were made to study cross-reactivities of the antibody preparations.

## MATERIALS AND METHODS

### Antibody Production

Antibodies were generated using six female New Zealand White rabbits. Each rabbit weighed approximately seven pounds on receipt and they were allowed to acclimatize to their holding area for 26 days before being used for antibody production. Two rabbits were used for each type of antibody. Antibodies were generated against three *E. coli* enzymes: ß-glucuronidase (Sigma Chemical Co., St. Louis, MO, product number G7896), glutamate decarboxylase (Sigma, product number G3757), and tryptophanase (Sigma, product number A6007). One ml of phosphate buffer (0.01 M $NaH_2PO_4 \cdot H_2O$, pH 7.2) containing 100 $\mu$g of an enzyme preparation was emulsified with 3 ml of complete Freund's adjuvant (Difco Laboratories, Detroit MI, product number 0638-60-7). The immunogen/adjuvant mixture was injected intradermally along the shaved back of the rabbit (19, 24, 49); each rabbit received approximately thirty to forty 0.025-ml injections. Thirty days later, fresh enzyme preparations were administered to boost specific anti-enzyme antibody levels. The second and subsequent immunogens consisted of 100 $\mu$g of appropriate enzyme suspended in 1 ml of 0.01 M phosphate buffer (pH 7.2) emulsified in 3 ml of incomplete Freund's adjuvant (Difco Laboratories, Detroit MI, product number 0639-59). The second and subsequent immunogens were used for approximately thirty to forty intradermal injections per rabbit.

Two weeks after the booster injections, whole blood was collected for purification of specific anti-enzyme antibodies. Blood was collected from anaesthetized rabbits by cardiac puncture and vacuum collection (Vacutainer System, Becton-Dickenson, Rutherford, NJ). The rabbits were anaesthetized to a surgical level with 1 ml of 1:3 acepromizine and ketamine. Approximately 30 mls of blood were collected and allowed to clot at room temperature for approximately 2 h. Cellular components were removed from the antisera by centrifugation of the clotted blood at approximately 1000 × *g* for 15 min (Sorval Instruments, DuPont Co., Newton CT,

model RT6000B) at 10°C. The antisera were transferred to labeled polypropylene centrifuge tubes (Corning product number 25319 or similar) and stored between -35 to -90°C until use.

The rabbits were subsequently given a booster dose of antigen approximately every month. Collection of whole blood followed two weeks after boosting, as described previously, for one year after which the rabbits were euthanized.

After six months, one rabbit, being used for anti-glucuronidase antibody production, expired. A male New Zealand White rabbit was acquired and another immunization series was started. Owing to previous problems in preparing homogenous emulsions with enzyme preparations and the difficulty in accurately performing inoculations caused by immunogen/adjuvant viscosity, an alternate adjuvant was used. One ml of Ribi adjuvant (Ribi ImmunoChemical Research, Hamilton MT, product number R730) was mixed with 0.78 mg of ß-glucuronidase according to Ribi recommendations. Briefly, the adjuvant was warmed to 45°C, 2 mls of ß-glucuronidase in 0.01 M phosphate buffer (pH 7.2) were injected into the vial and the vial was agitated on a vortex mixer for 3 min. The rabbit was immunized with 0.3 ml intradermally (0.05 ml at six sites along the back), 0.4 ml intramuscularly (0.2 ml in each hind leg), 0.1 ml subcutaneously (in the neck region), and 0.2 ml intraperitoneally. A booster inoculation was administered 21 days later using 1.02 mg of ß-glucuronidase in Ribi adjuvant as previously described. The rabbit was bled 26 days later and then established on the monthly boost/bleed cycle with the remaining five rabbits.

**Antibody Purification**

Antibodies against three *E. coli* enzymes were purified from stored antisera. The immunoglobulin G (IgG) fraction of each serum was separated from other serum proteins by one of three methods; protein A chromatography, antigen-specific affinity chromatography, or diethylaminoethyl (DEAE) column chromatography.

***Protein A antibody purification***

For purification by using protein A, the serum was diluted 1:1 with 1 M Tris buffer (pH 8.0) and clarified by passage through a 0.22- or 0.45-$\mu$m cellulose acetate syringe filter. The clarified serum (5 to 20 ml per application) was passed through a protein A-Sepharose (Sigma, product number P2545; binding capacity of approximately 100 mg of IgG) 8 cm x 1.25 cm column equilibrated with 100 mM Tris buffer (pH 8.0). The eluate was monitored at 280 nm with an Isco model UA-2 spectrophotometer (Instrumentation Specialties Co., Inc., Lincoln, NE) equipped with a flow cell. Passage of unwanted serum proteins was seen as a rise then fall of absorbance values with continuous flow of 100 mM buffer through the column (approximately 15 to 20 ml of buffer after serum application). After the absorbance returned to the base line, the wash was continued for another 10 to 15 ml followed by approximately 20 ml of 10 mM Tris buffer (pH 8.0). IgG was eluted from the column with 0.1 M glycine (pH 2.8). When the absorbance started to increase, the fraction containing IgG was collected in a polypropylene centrifuge tube containing approximately 2 ml of 1 M Tris buffer (pH 8.0) until the absorbance returned to the base line (approximately 15 ml). The pH was adjusted to 7-8 with 1 M Tris (pH 8.0). Alternatively, IgG was eluted from the column with a sodium acetate solution (2.5 ml glacial acetic acid, 4.5 g NaCl, 0.2 g $NaN_3$, quantity sufficient to make 500 ml with $dH_2O$, pH 2.8).

***ImmunoPure® gentle antibody elution from protein A***

As an alternative to standard protein A purification, ImmunoPure® gentle antibody elution (Pierce, Rockford, Il, product number 21014) was used. This procedure is termed the "gentle method" for the remainder of this dissertation. Antibodies were purified according to instructions from the manufacturer. Briefly, this entailed diluting rabbit serum 1:1 with binding buffer (buffers were supplied with the kit). Diluted serum was applied to an equilibrated 1-ml protein A column and allowed to flow completely into the gel. The column was washed with approximately 15 ml of

binding buffer. IgG was dissociated from the protein A and eluted with 5 ml of ImmunoPure® gentle Ag/Ab elution buffer. The antibody fraction was extensively dialyzed before further use.

***Antigen-specific affinity chromatography antibody purification***

Antigen-specific affinity chromatography columns (Pierce, product number 44890) were prepared according to the manufacturer's instructions. Briefly, activated Sepharose 4B in polypropylene chromatography columns was mixed with 5 to 10 mg of enzyme in coupling buffer (0.1 M phosphate, pH 7). A reducing solution (0.2 ml; sodium cyanoborohydride) was added. The columns were mixed by gentle rocking on a Thermolyne Speci-Mix (model M26125) for 2 h followed by 4 h stationary. The columns were blocked with 2 ml of quenching buffer (Tris-HCl, pH 7.4) and 0.2 ml of reducing solution for 30 min before washing and use. Initially, the columns were prepared by using the same commercial enzymes used for antibody production. Later, preparations of tryptophanase and glutamate decarboxylase, believed to be of better purifty, were used. (They were gifts from Dr. David Metzler, Department of Biochemistry and Biophysics, Iowa State University.)

For antigen-specific affinity chromatographic purification, the serum was first diluted 1:1 with 1 M Tris buffer (pH 8.0) and clarified by filtration through a 0.22- or 0.45-$\mu$m syringe filter. Serum (1 to 2 ml) was added to an antigen-specific affinity chromatography column that had been equilibrated with 100 mM Tris buffer (pH 8.0). When the sample had completely entered the gel bed, 0.2 ml of buffer was added and allowed to enter the gel bed, at which time the bottom cap was placed on the column. One ml of buffer was added to the column and the column was incubated at room temperature. After one hour (occasionally longer), the column was washed with approximately 16 ml of 100 mM Tris buffer (pH 8.0). Affinity specific immunoglobulins were eluted from the column with 0.1 M glycine (pH 2.8). Either individual 1-ml fractions, or fractions 2 through 6 in bulk, were collected and neutralized with 1 M Tris buffer.

*DEAE Affi-Gel® Blue antibody purification*

DEAE Affi-Gel® Blue columns (Bio-Rad, Richmond, CA, product 732-3037) were used as recommended by the manufacturer. Briefly, samples were first desalted by using the Econo-Pak 10DG columns and the application buffer (for rabbit immunoglobulins) supplied with the kit. The eluate from the desalting column was applied to an equilibrated DEAE Affi-Gel® Blue column followed by 20 ml of application buffer. Fractions were collected and those containing IgG, as determined by absorbance at $A_{280}$ (6, 19), were combined. Antibodies purified by this method are called as DEAE antibodies for the remainder of this dissertation.

*Dialysis and storage*

The immunoglobulins were dialyzed against 0.03 M Tris/azide buffer (0.03 M Tris, 0.05% sodium azide, pH 7.8), unless otherwise noted. Quantities of immunoglobulins recovered were estimated by using $A_{280}$ values and an extinction coefficient of 1.4 (6, 19). As necessary, the immunoglobulins were concentrated by using either a 15- or 2-ml capacity Centraprep 30 unit (Amicon Division, W.R. Grace and Co., Beverly, MA, product numbers 4306 and 4208, respectively). Working stocks of immunoglobulins were stored at 4°C until used. Immunoglobulins more than the immediate need were stored at -30 to -95°C.

**Characterization of Antibodies**

*Polyacrylamide gel electrophoresis*

A nondenatured polyacrylamide gel electrophoresis (PAGE) and a denatured PAGE was prepared for each of four types of immunoglobulin preparations. PAGE procedures were conducted according to the instruction Manual (89-0451 789 dated 1989) for the Bio-Rad Protean IIxi Slab Cell electrophoresis unit (Bio-Rad Laboratories, Richmond, CA). The gels were discontinuous gels on the basis of the method of Laemmli (2, 19). Briefly, two 16-cm glass plates were spaced 1 mm apart in sandwich clamps, aligned and placed in the casting stand. Activated,

degassed 12% acrylamide/bis monomer solution was allowed to fill the space in the glass sandwich to a point 1.5 cm below the bottom of the comb assembly wells. The gel was overlaid with $dH_2O$ and allowed to polymerize for 45 to 60 min or overnight. After polymerization, the top of the gel was rinsed and dried before casting the stacking gel. An activated, degassed 4% stacking gel was poured to the top of the glass sandwich with the comb assembly in place and allowed to polymerize for 30 to 40 min. The comb assembly was removed, and the wells were rinsed free of unpolymerized reagents and dried. The glass sandwich with gel and a buffer dam was mounted in the cooling core and the unit was placed in the tank assembly with buffers. Approximately 10 $\mu$l of sample in sample buffer (6 ml of 20% aqueous sodium dodecyl sulfate, 3 ml of glycerol, 2.4 ml of 1 M Tris-HCl (pH 6.8), 15.6 mls of $dH_2O$, and a few grains of bromophenol blue) were underlayed in each well. The lid was put in place and the unit connected to an Isco electrophoresis power supply (Isco, Lincoln, NB, model 494). A constant 16 mA was applied to the gel until samples entered the separating gel; Then the current was increased to 24 mA and held constant. The samples were allowed to migrate through the gel until the leading edge was within 1 to 2 cm of the bottom edge of the gel. On completion of electrophoresis, the gel was removed from the unit and fixed in an aqueous solution of 40% methanol and 10% acetic acid until further use.

***Ouchterlony immunodiffusion analysis***

Ouchterlony immunodiffusion (8, 9, 23, 29) slides were prepared on 7 mil 10 x 3.75 mm electrophoresis film (Sigma product number E 1380) with a 0.5" header on one end. Six ml of immunodiffusion agar (Bio-Rad Laboratories, product number 170-3002) were added to each slide with a pipette. After hardening, 4-mm diameter wells were cut by using a Grafar Auto-Gel punch assembly (Grafar Corp., Detroit, MI). The wells were spaced 6 mm, edge to edge, from the center well. Either antibody or antigen (20 $\mu$l) was added to each well. The slides were incubated in

humidified petri dishes for 24 to 48 h. After incubation, the slides were soaked in 0.3 M NaCl overnight and then were stained with Coomasie blue stain (Bio-Rad Laboratories, product number 500-0001) for 20 min. The slides were destained with two changes of dH2O until the background was clear. Precipitant bands stained as blue bands between wells.

***Western blot analysis***

Reactivity of each of the antigen affinity purified antibody preparations (anti-tryptophanase, anti-glutamate decarboxylase, and anti-glucuronidase) toward the electrophoretically separated commercial enzymes was determined by Western blot analysis. A nondenatured PAGE was made essentially as previously described. The three commercial enzymes and suitable controls (ß-galactosidase, Sigma product number G5635, L-glutamate decarboxylase from *Clostridium perfringens*, sigma product number G2376, and ß-glucuronidase from *Helix pomatia* [snail], Sigma product number G1512) were used as samples. The first seven and second seven wells of the gel were loaded in such a manner as to provide a duplicate pattern for each gel run. After completion of electrophoretic separation of the enzymes and controls, the gel was cut in half; one-half was used for general protein staining and the other half for Western blotting.

Western blotting was performed essentially as described by Gershoni and Palade (15) and instructions supplied with an Immun-blot kit (Bio-Rad Laboratories, product number 170-6450). Briefly, the gel was sandwiched between an approximately 6 x 13 cm sheet of nitrocellulose and filter paper (2) and placed in the electroblotting unit (Transphor Power-Lid, model TE50, Hoefer Scientific Instruments, San Francisco, CA). Ten amps at a constant 14 volts were applied. After approximately 18 h, the nitrocellulose sheet was transferred to a heat-sealable bag for the remaining procedures. The nitrocellulose sheet was blocked for one hour with 3% gelatin in Tris buffer. The nitrocellulose sheet was then washed and incubated for 18 h with a 1:1000 dilution of primary antibody (approximately 2.8 $\mu$g for anti-glutamate decarboxylase, 1 $\mu$g for

anti-tryptophanase, and 1 $\mu$g for anti-glucuronidase). After incubation, the primary antibody was removed, the membrane washed, and the secondary antibody (goat anti-rabbit with conjugated alkaline phosphatase) added. The secondary antibody was incubated with the nitrocellulose sheet for approximately 2 to 3 h before being removed. The substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were added and allowed to react until band development appeared to be complete (15 to 30 min). The reaction was stopped by removing the nitrocellulose sheet from the bag and washing the membrane in $dH_2O$.

Besides the four Western blots of the commercial enzymes, a gel was run by using all three enzymes separated by a sample containing bovine serum albumin (BSA; Sigma product number A-7030). After PAGE and electroblotting, the blot was cut vertically so that each blot strip contained two of the blotted gel lanes; one lane contained an enzyme and a second lane contained a BSA sample. Each blot section was incubated with primary antibody against the enzyme and processed as described previously.

***EIA analysis***

*Preparation of conjugated enzymes*

Alkaline phosphatase-antibody conjugates were prepared essentially as described by Voeller et al. (50). Approximately 1.4 mg of antibody in 1 ml of PBS (0.05 M, pH 7.4) was added to 5 mg of alkaline phosphatase (type VII-T, Sigma product number P6774). Two microliters of 25% glutaraldehyde (Sigma, product number G-5882) were added and the preparation was incubated at room temperature for 2 h. The preparation was dialyzed overnight at 4°C against PBS (0.05 M, pH 7.4). The buffer was changed to 0.05 M Tris buffer (pH 8.0, containing 1 mM of MgCl) and the conjugated antibodies were again dialyzed overnight at 4°C. After dialysis, BSA and sodium azide, enough to yield 1.0 and 0.2% solutions respectively, were added to the conjugated antibodies. The preparations were stored at 4°C.

*Preparation of dot enzyme-linked immunosorbent assays*

An 8 × 12 cm membrane sheet (UltraBind™ US-450, Gelman Sciences, Ann Arbor, MI, product number S80245) was prewet with PBS (0.05 M, pH 7.2) and placed in a vacuum dot-blot apparatus (Bio-Dot™, Bio-Rad Laboratories, product number 170-6545). Commercial enzymes (2 mg/ml glutamate decarboxylase, Sigma, product number G3757 and 0.1 mg/ml ß-glucuronidase, Sigma, product number G7896), were reconstituted with 0.05 M PBS. To each well in columns two through twelve, 50 µl of 0.05 M PBS was added. To each well in column one, 100 µl of an enzyme was added. Serial dilutions of enzymes were completed for columns two through twelve (except column three was skipped to serve as a control) by taking 50 µl from one column and mixing it with the buffer in the next column. To column three, 50 µl of 1% BSA (in 0.05 M PBS) was added. The solutions were allowed to react with the membrane for 2 min before a gentle vacuum was applied to the membrane through the apparatus. Approximately 0.05 ml of 1% nonfat dried milk (Sigma, product number M-7409) was added to each well and the vacuum was reapplied. The membrane was washed three times with 0.05 ml of PBS containing 0.01% Tween 20. Alkaline phosphatase conjugated antibodies were diluted and added to the wells according to the schemes shown in Figures 1 and 2. The conjugated antibodies were allowed to react for approximately 5 min before being gently removed by vacuum. Unreacted antibodies were removed with three 0.1-ml applications of wash solution (0.05 M PBS, 0.01% Tween 20). The membrane was removed from the apparatus, placed in a pouch that was sealed by heat for each of the following steps. The membrane was washed for 15 min in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) with 0.12% nonfat dried milk added. The first wash solution was removed and the membrane was washed three times with 15 ml of TTBS. After the final TTBS wash, the membrane was washed in TBS (20 mM Tris, 500 mM NaCl, pH 7.5). The TBS solution was removed and 15 ml of substrate (BCIP/NBT, Bio-Rad Laboratories, component of product

number 170-6450) were added. After approximately 5 min, the membrane was removed and washed in $dH_2O$.

# RESULTS AND DISCUSSION

## Antibody Production and Purification

Kaspar (31) developed enzyme capture and coagglutination methods to test cell lysates for the presence of specific enzymes. Although the methods were described as sensitive and specific, they have not been adopted for routine use, most likely because they are inconvenient to carry out. in the original work, Kaspar (31) produced antibodies to four *E. coli* enzymes: ß-glucuronidase, ß-galactosidase, glutamate decarboxylase, and tryptophanase. The antibodies were produced in rabbits using commercial enzyme preparations in Freund's adjuvants. Anti-enzyme serum titers were not determined. All the antibody preparations, except anti-tryptophanase, performed satisfactorily in Kaspar's assays (31). The anti-tryptophanase assays were unable to detect tryptophanase in *E. coli* cell lysates. Kaspar concluded that this could be the result of either of two possibilities. The first was that the anti-tryptophanase titers were to small because the tryptophanase used for antibody production was not as pure as the other enzyme preparations. The second possibility was that tryptophanase production was repressed in the enzyme induction broth being used for growing the cell lysates.

To avoid problems that Kaspar encountered in his work, some minor changes were made in the production of the antibodies. Rabbits were immunized using a different protocol that should have produced better antibody preparations. The change to several smaller intradermal injections has been described as making higher affinity antibodies (10, 49). To improve on the anti-tryptophanase production, a tryptophanase was selected as the immunogen that was thought purer than the enzyme preparation Kaspar had used.

Initial efforts to develop latex agglutination tests resulted in extensive false-positive reactions, with both cell lysates and commercial enzyme preparations, including negative controls. Because antibody preparations functioned well in Kaspar's systems (31), similar preparations

should function satisfactorily in latex agglutination tests. Because one major difference between latex agglutination and coagglutination of enzyme capture methods is adsorption of antibodies on the latex, initial studies were performed on adsorption of antibodies to latex particles (described in part 2 of the dissertation) and the effects of blocking agents on assay sensitivity and specificity (described in part 3). Another likely cause of the false-positive reactions (which we now believe was the major problem, so it is reported first) is the antibody preparations themselves. Enzyme induction, cell lysis, and interpretation of the results were not considered problem areas.

**Electrophoretic analysis of antibody proteins**

One major variable was the method of antibody preparation; antigen-specific affinity chromatography purified antibody preparations were used in this project whereas Kaspar had used protein A purified antibody preparations (31). Antigen-specific affinity chromatography purified antibodies were used because they should contain a greater fraction of specific antibodies than protein A purified antibodies. It is known, however, that isolation methods induce changes in antibodies (34) and by changing the isolation method, a change in assay results could occur. Furthermore, Limet et al. (33) theorized that rabbit rheumatoid factors present in IgG preparations caused spontaneous agglutination. Antibodies purified by antigen affinity chromatography, as used in this study, should have been free of contaminating rheumatoid antibodies, and were not considered part of the problem. When there were no changes in efficacy of the latex agglutination assays when antigen-specific affinity purified antibody preparations were replaced with protein A purified antibodies, other methods of isolation were tried. Even with the other purification methods, there was no difference in false-positive reactions, now being called nonspecific agglutination, because of different antibody purification methods (data presented in part 2).

The different immunoglobulin preparations were evaluated by polyacrylamide gel electrophoresis (PAGE) to determine the presence of extraneous nonimmunoglobulin proteins.

(Not all bands discussed in the following sections can be observed on the prints made of the photographs of the gels.)

***Nondenaturing conditions***

Antibodies to tryptophanase purified by all methods exhibited a major band at approximately 147 kDa and a minor band at approximately 54-kDa (Figure 3). The 54-kDa bands were more discernible by observing the gel then they are on the photographic prints of the gels. All except the gentle method had an additional band at approximately 67 kDa. The DEAE purified antibodies had three additional bands corresponding to 75, 66 and 52 kDa.

Antibodies to glucuronidase purified by all methods exhibited a major band at approximately 147 kDa and a minor band (is very faint on the photographic print) at approximately 54 kDa (Figure 3). DEAE and antigen-specific affinity chromatography purified antibodies had four additional bands corresponding to 75, 67, 66 and 52 kDa, similar to anti-tryptophanase.

The major band in gels of anti-glutamate decarboxylase was calculated to have a molecular mass of 143 kDa, slightly less than anti-tryptophanase and anti-glucuronidase, but within experimental error (Figure 4). Similar to anti-tryptophanase and anti-glucuronidase, a minor band at approximately 68-kDa was detected in all antibody preparations. Other bands observed included a 54-kDa band in protein A (lane B), the gentle method (lane D), and Kaspar's protein A preparations (lane F), 82- and 44-kDa bands in antigen-specific affinity chromatography purified preparations. Two additional bands appeared at 75, and 67 kDa in the DEAE-purified preparations.

In general, all PAGE preparations revealed a significant band at approximately 146 kDa that probably represents the intact rabbit IgG molecule. The minor band at approximately 54 kDa is most likely the result of some denaturation of the IgG molecule into fragments; the heavy chain would be at 54 kDa. Light chain fragments most likely failed to be separated at the low molecular weight front. Other bands represent either contaminating proteins not removed by the antibody

purification processes or other isotypes of immunoglobulins including fragments of IgM. This is possible especially for antigen-specific affinity chromatography purification because it is not specific for IgG but rather for any immunoglobulin class present that will bind antigen. Except in the DEAE-purified antibodies, the concentrations of non-IgG proteins were considerably less than those of IgG and were not considered significant. Gels of DEAE-purified antibodies revealed as many as four additional bands, some of which contained substantial quantities of protein and might be considered significant contamination.

***Denaturing conditions***

Because the nondenatured PAGE assays revealed the presence of more protein bands than should be present in homogeneous antibody preparations, fully denatured antibody preparations were subjected to PAGE. As expected, no major bands were observed at approximately 147 kDa and the major band, for all preparations, was at approximately 59 to 61 kDa (Figures 5, 6, and 7).

Protein A preferentially binds immunoglobulins, especially those of class IgG (17, 40). Antibodies purified with protein A and the gentle method displayed a homogeneity consistent with a single subclass of immunoglobulin (Figures 5, 6, and 7).

Antigen-specific affinity chromatography purification is not as specific toward a single class of immunoglobulin as is the protein A method. Any class of immunoglobulin that will bind to the antigen will later be eluted off the solid support. This is demonstrated in the denatured PAGE of anti-glucuronidase (Figures 5, 6, and 7). Antigen-specific affinity chromatography purification of anti-glucuronidase revealed three additional bands that probably correspond to denatured chains of other immunoglobulin classes, such as IgM or IgD.

Denatured PAGE gels of DEAE-purified antibodies (Figures 5, 6, and 7) revealed five to eight distinct bands. The bands obtained did not correspond with albumin, the major serum

protein. DEAE purification is intended to bind the albumin fraction and other negatively charged proteins, allowing the neutral or positively charged immunoglobulin fraction to pass through (24). Other serum proteins also pass through DEAE columns. The manufacturer of the columns stated that residual contamination of IgG with transferrin is normal (5). Transferrin, an iron transporting protein, has a molecular mass of 76.5 kDa (43) that is consistent with a band obtained on the PAGE of the DEAE-purified antibodies examined here. In addition to transferrin, other proteins such as complement proteins (C1, molecular mass of 86 kDa; 43), plasminogen (molecular mass 87 kDa; 43), or hexokinase (molecular mass 97 kDa; 47) might account for other protein bands that appeared in the PAGE gels.

The major bands at 59 to 61 kDa are consistent with the molecular mass attributed to IgG heavy chain fragments (11, 18). IgG light chain fragments were not resolved from the protein front in these gels. Antibodies purified by the DEAE method had at least four bands; besides the heavy chain fragments, bands appeared at approximately 93, 82, and 76 kDa. These fragments, although the calculated molecular masses differed slightly, are probably the same proteins present in the nondenatured PAGE, suggesting that the proteins are not immunoglobulin fragments.

This paragraph is presented to describe important bands that would be observed on the denatured PAGE. Some analogies with the human immunoglobulins are included because rabbit immunoglobulin primary structures bear many similarities. Normal human immunoglobulin is composed of nine known different types or subclasses of immunoglobulins: $IgG_1$, $IgG_2$, $IgG_3$, $IgG_4$, IgM, $IgA_1$, $IgA_2$, IgD, and IgE (18, 19). Molecular masses of whole molecule immunoglobulins range from 970 kDa for IgM to 146 kDa for $IgG_{1,2,4}$. The immunoglobulins are composed of four subunits that can be denatured into components with molecular masses ranging from 72 to 51 kDa for IgE and $IgG_{1,2,4}$ heavy chains, respectively, and from 32 to 21.5 kDa for IgM and IgE light chains, respectively (18, 19). Rabbit immunoglobulins, however, are made up of only four

subclasses: IgG, IgM, IgA, and IgE (38). Unlike human immunoglobulins, rabbit IgG exists as a single subclass, however, considerable heterogeneity, with respect to overall charge, exists (16, 38). Although IgG composes over 90% of the total immunoglobulin in rabbit serum (38), the immunoglobulin fraction constitute only 4.3 to 11.8% of the total serum proteins (20, 35, 47). In other animals, such as sheep or goats, a much greater percentage (15 to 26% and 13 to 31%, respectively) of their serum proteins consists of immunoglobulins (20, 35, 47). Unlike human IgG, the two chains of rabbit IgG are held together by only a single disulfide bond (38). The molecular mass of rabbit IgG has been reported to range from 145 to 188 kDa (42, 44). Results of this study are more consistent with the smaller molecular mass of 145 kDa. Overall, rabbit immunoglobulin molecular masses are considered similar to human immunoglobulin.

In summary, the results of denaturing PAGE assays indicated that all four antibody purification methods should result in the preparation of suitable antibody preparations for use in latex agglutination tests.

**Ouchterlony**

The initial development of the latex agglutination assay included false-positive reactions when commercial enzymes, other than those used to elicit antibody production, were used as negative controls. To evaluate if the immunoglobulin preparations contained antibodies cross-reacting with the enzymes used as negative controls, sixty Ouchterlony immunodiffusion tests were run with combinations of antibodies and antigens (Table 1). The antibodies were purified by using the four different methods described in Materials and Methods, and the antigens were commercial enzyme preparations. Reactions and cross-reactions of antibodies and antigens were seen as one or more visible bands or lines between the two reactants.

*Tryptophanase*

Using commercial tryptophanase preparations at an estimated concentration of 1 mg/ml, levels of endpoint titration to antibody concentrations were higher than those obtained with anti-glucuronidase. Protein A-purified antibodies again had a range of endpoints, from 1 to 0.5 mg/ml in repeatable tests (Table 1 and Figure 8). Antibodies from the gentle method had an endpoint of 0.5 mg/ml (Table 1). With antigen-specific affinity chromatography anti-tryptophanase, an endpoint of 0.25 mg/ml was observed. DEAE-purified antibodies did not form a precipitate with any concentration of antibody or antigen tested. When the antibody level was held constant at 1 mg/ml and the concentration of commercial enzyme was varied, protein A and antigen-specific affinity chromatography purified antibody both had endpoints of 0.125 mg/ml. Antibodies gently eluted had an endpoint titration of 0.0625 mg/ml. When testing anti-tryptophanase against the other commercial enzymes, antibodies purified by protein A had precipitation reactions against both glucuronidase and glutamate decarboxylase.

*Glucuronidase*

Using commercial glucuronidase enzyme at an estimated concentration of 1 mg/ml, protein A-purified antibodies developed a precipitation band when endpoint concentrations of 0.5 to 0.0625 mg/ml of antibody were used (Table 1 and Figure 9). When gentle methods and DEAE purification were used, an endpoint titration of 0.5 mg/ml of antibody was observed. If antigen-specific affinity chromatography purified antibodies were used, an endpoint titration of 0.0625 mg/ml was observed. Holding the antibody constant at 1 mg/ml and varying the concentration of commercial enzymes, protein A-purified antibody had endpoint titrations of 0.0625 to 0.03125 mg/ml. DEAE and antigen-specific affinity chromatography purified antibodies had endpoint titrations of less than 0.03125 mg/ml. The gentle method of antibody elution resulted in an endpoint titration of 0.0625 mg/ml. In no tests of anti-glucuronidase antibodies against either

commercial tryptophanase or glutamate decarboxylase at any concentration tested, did a visible precipitin band form.

***Glutamate decarboxylase***

Antibodies to glutamate decarboxylase from the four different purification methods as well as a previously purified antibody by another investigator (32) were also tested. When a commercial glutamic acid decarboxylase at an estimated concentration of 1 mg/ml was used, the levels of endpoint titration for protein A purified and gently eluted antibodies were 0.25 mg/ml (Table 1 and Figure 10). Both antigen-specific affinity chromatography and previously purified antibodies had endpoint titrations of 0.5 mg/ml. Antibodies isolated with the DEAE method resulted in an endpoint of only 1 mg/ml. Holding the antibody constant at 1 mg/ml and varying the concentration of commercial enzyme preparation resulted in a titration endpoint for all the antibodies of less than 0.0625 mg/ml. When testing antibodies to glutamate decarboxylase for reactions to other commercial enzymes, all the antibodies except those gently eluted, reacted with tryptophanase but not glucuronidase.

Ouchterlony immunodiffusion has been used for many years to detect immunological reactions. Although it is not highly sensitive, it is relatively simple to do and could provide the types of information necessary to evaluate the antibody preparations used in this project. Although the tests did prove that the antibody preparations were functional at relatively low titers, the Ouchterlony method was neither as precise as needed nor readily reproducible. When using protein A-purified antibodies and their respective enzymes, results of titration endpoints often varied up to five two-fold dilutions. Furthermore, in reactions designed to indicate cross-reactions with the nontarget enzymes, the results often were contradictory. Also, there should have been no significant differences in the cross-reactivities of antibodies purified with protein A and those from

the gentle method. This was not so, however, and another approach to detecting cross-reactivity was determined necessary.

## Western Blotting

Because of the difficulty in interpreting and drawing conclusions from the Ouchterlony immunodiffusion work, another method of detecting cross-reactivities was needed. Western blotting, normally a technique using antibodies for the analysis of other proteins, was performed in a reversed format to evaluate antibodies and not other types of proteins. The Western blot analyses revealed that the enzyme preparations were not as homogeneous as previously thought. A denatured PAGE of the commercial enzymes is shown in Figure 11 to serve as a basis for comparisons to the Western blots.

### *Anti-tryptophanase primary antibody*

When anti-tryptophanase antibody was used as the primary antibody, a blot was obtained that had over 40 distinguishable bands in the lane containing commercial tryptophanase (Figure 12, lane C). The most intense bands were at approximately 55 kDa. This would closely approximate the monomeric size of tryptophanase (46 to 55 kDa; 36). Other bands within the tryptophanase lane probably represent contaminating proteins and possibly enzyme fragments. Since the commercial enzyme preparation used for the Western blot also was used to produce antibodies, detection of these proteins is expected.

Reactivity with proteins in the commercial preparation of *E. coli* glutamate decarboxylase resulted in fifteen distinguishable bands (Figure 12, lane A). The most intensely reactive of these bands were at molecular masses of approximately 54 and 26 kDa. The former band likely represents contaminating tryptophanase in the glutamate decarboxylase preparation whereas the latter probably represent protein fragments and other small molecular mass proteins not removed from the commercial preparation during purification. In the molecular mass range of monomeric

glutamate decarboxylase (46.5 to 50 kDa; 46, 48), two bands were present that could represent cross-reactivity of the anti-tryptophanase with glutamate decarboxylase enzyme. Remaining reactive bands most likely represents other cross-reactive impurities in the glutamate decarboxylase enzyme preparation.

Reactivity with the proteins in the commercial preparation of glucuronidase (Figure 12, lane B) resulted in eight distinguishable bands. Of these, three were very strongly reactive and one was broad and diffuse. Both the diffuse band and one of the strongly reactive bands were between 75 and 84 kDa. The other strongly reactive band within the glucuronidase lane was at a molecular mass of approximately 36.6 kDa. *E. coli* has well over 100 different proteins, ranging from 30 to 80 kDa (41). Differentiation of the reactive protein bands was beyond the scope of this study.

Reactivity with the control proteins resulted in ten slightly reactive bands with the galactosidase (Figure 12, lane D) and nine slightly reactive and two very reactive bands in the *C. perfringens* glutamate decarboxylase lane (Figure 12, lane E). The broad and diffusely reactive band at 116 kDa with galactosidase corresponds to the molecular mass of monomeric galactosidase (19). The identities of the other reactive bands are not known nor could they be readily determined.

For the most part, the cross-reactions of anti-tryptophanase were mainly within the enzyme preparations used for immunizations and did not extend as strongly to other enzyme preparations used as controls. The reactions revealed very little reactivity with the *E. coli* ß-galactosidase preparation used as a control. Considering the extensive heterologous nature of the ß-galactosidase preparation from the commercial source (Figure 11), equivalent cross-reactions from the anti-tryptophanase should be more evident. If the ß-galactosidase control had shown more reactivity with the antibody preparations, the antibody preparations would have probably been considered more seriously impaired and probably would have been redone. Consequently, the cross-reactivity of the antibody preparations was viewed as not as significant as it probably

was. In retrospect, the results of the Western blotting should have suggested that better enzyme preparations were required and new antibody preparations, using more specific antigens, should have been made.

***Anti-glutamate decarboxylase primary antibody***

When anti-glutamate decarboxylase antibody was used as the primary antibody, a blot was obtained that had over 23 distinguishable bands reactive with glutamate decarboxylase (Figure 13, lane A). The most intense bands were at approximately 78, 55, 35, 27, and 26 kDa. The monomeric mass of *E. coli* glutamate decarboxylase is approximately 52 kDa (46, 48). Bands, other than the 55-kDa band, within the glutamate decarboxylase lane probably represent contaminating proteins. Because the commercial enzyme preparation was used to produce antibodies, reactivity with these proteins would be expected.

Reactivity with proteins in the commercial preparation of glucuronidase (Figure 13, lane B) resulted in eleven distinguishable bands. Two of the most reactive bands were at approximately 81 and 72 kDa, the molecular mass of glucuronidase. Reactivity with proteins in the commercial preparation of tryptophanase (Figure 13, lane C) resulted in four distinguishable bands. The most reactive band was at approximately 36.8 kDa and could be one of several *E. coli* proteins. There was no significant cross-reactivity with the galactosidase (Figure 13, lane D) control but the *C. perfringens* glutamate decarboxylase control (Figure 13, lane E) did have a reactive band at approximately 76 kDa. Although the reactive band within the *C. perfringens* glutamate decarboxylase lane was at the same approximate molecular mass as similar reactive bands in all three commercial enzyme preparations, it was significantly heavier than the molecular masses of *E. coli* or *C. perfringens* glutamate decarboxylase (13).

Similar to the interpretation of the anti-tryptophanase Western blot results, the anti-glutamate decarboxylase preparation was more strongly cross-reactive with the enzymes used for

immunizations than with the control enzyme preparations. The lack of significant reactivity with the control enzyme preparations lead to the belief that the cross-reactivities of the antibody preparation was not as consequential as it was later discovered.

***Anti-glucuronidase primary antibody***

Use of anti-glucuronidase antibody as the primary antibody yielded a blot that had over 29 separately distinguishable bands in the lane containing commercial *E. coli* glucuronidase (Figure 14, lane B). The most intense band was at approximately 76 kDa. This would closely approximate the monomeric size of 68 to 82 kDa of glucuronidase reported by others (12, 14, 22, 39). Other bands within the glucuronidase lane most likely represent contaminating proteins. Because the commercial enzyme preparation was also used to produce antibodies, reactivity of these proteins is expected on a Western blot.

Reactivity with proteins in the commercial preparation of glutamate decarboxylase (Figure 14, lane A) resulted in sixteen distinguishable bands. The three most intensely reactive bands were at a molecular mass of approximately 76, 52, and 26 kDa. The 76-kDa band likely represents contaminating glucuronidase in the glutamate decarboxylase preparation. The 52-kDa band could be cross-reactivity with either tryptophanase, glutamate decarboxylase, or another *E. coli* protein of that molecular mass. The 26-kDa band probably represents protein fragments and other small molecular mass proteins not removed from the commercial preparation during purification.

Reactivity with the proteins in the commercial preparation of tryptophanase (Figure 14, lane C) resulted in four distinguishable bands. The first band was at approximately 91 kDa. This could represent a cross-reactive dimeric form of tryptophanase that was not fully denatured or it could be a contaminating protein that was present in both the glucuronidase preparation used for the immunizations and the tryptophanase enzyme preparation. Bands at 52 and 26 kDa are similar to those obtained in the reaction with glutamate decarboxylase and probably represent similar

contaminating proteins.

Reactivity with the control proteins (Figure 14, lanes D and E) resulted in two very slightly reactive bands with galactosidase and no detectably reactive bands with *Helix pomatia* (snail) glucuronidase (bands may not be visible on the photograph). The broad and diffusely reactive band at 116 kDa with galactosidase corresponds to the molecular mass of monomeric galactosidase (19); thus it is likely that the glucuronidase preparation used for immunization contained a trace of ß-galactosidase. Lack of reactions with *H. pomatia* glucuronidase revealed that *E. coli* and *H. pomatia* glucuronidase did not share common immunologic epitopes.

Again, like the results from the anti-tryptophanase and anti-glutamate decarboxylase Western blot results, the cross-reactions with the anti-glucuronidase were confined predominately to the enzymes used for immunizations and they did not significantly extend to the control enzyme preparations. These results are consistent with, and support the supposition available at this time, that the cross-reactions were not seriously compromising the character of the antibody preparations. The antibody preparation's cross-reactivity within the enzymes used for immunizations, however, was significant and ideas on how to best deal with them were considered.

***Absorbed anti-glutamate decarboxylase primary antibody***

After observing the results of the first three Western blots, this Western blot was done to determine if cross-absorption of an antibody preparation with the other enzyme preparations could significantly reduce the cross-reactivity of the antibody preparation. When absorbed anti-glutamate decarboxylase antibody was used as the primary antibody, a blot that had over 19 separately distinguishable bands with commercial glutamate decarboxylase was obtained (Figure 15, lane A). This was a reduction of only four bands from the nonabsorbed anti-glutamate decarboxylase (Figure 15, lane A). The most intense bands (Figure 15, lane A) were at approximately 79, 58, 44,

and 22 kDa; this was similar to the results of the reactions with nonabsorbed anti-glutamate decarboxylase, indicating that adsorption did not result in significant loss of anti-glutamate decarboxylase activity.

Reactivity with proteins in the commercial preparation of glucuronidase (Figure 15, lane B) resulted in two distinguishable bands. In comparing this blot to the blot with the nonabsorbed antibody (Figure 13, lane B), this blot revealed a loss of several bands and significant loss of intensity in the band at 95.6 kDa. Reaction with the proteins in the commercial preparation of tryptophanase (Figure 15, lane C) resulted in eleven distinguishable bands. In comparing this blot to the blot with the nonabsorbed antibody (Figure 13, lane C), this blot revealed a significant gain of a band at approximately 28 kDa. Although absorbed anti-glutamate decarboxylase contained fewer cross-reacting antibodies than the unabsorbed antiserum, the absorbed preparation retained so many cross-reactivities that there was no difference of practical significance between the absorbed and nonabsorbed preparations.

***Anti-BSA cross-reactivity***

Because of the heterogenous mixture of proteins contained in the commercial enzyme preparations and the extent of the false-positive reactions, detection of any anti-BSA contamination in the antibody preparations was evaluated. BSA was being used to block any unreacted sites on the latex after immunoglobulin adsorption. Any anti-BSA in the antibody preparations would seriously compromise the antibody preparations when BSA was being used to block the latex.

A PAGE was run with all three enzymes separated by a sample containing 1 mg/ml BSA. BSA was used to block any unsaturated spots on the latex during sensitization of the latex with antibodies. This Western blot determination was made to insure that no anti-BSA cross-reactivity existed in the antibody preparations.

Each lane containing the commercial enzyme preparations had reactions consistent with

the Western blot results discussed previously (Figure 16). No lane containing BSA had any significant reaction with the primary antibodies. Therefore, cross-reactions within the latex system were not caused by a reaction of the antibodies with the BSA that was used in blocking the latex.

In general summary, the Western blots showed that besides extensive cross-reactions with the commercial enzyme preparations, the reactions within the immunizing enzyme preparation were often considerable, consisting of up to forty different antigenic proteins. Feng and Lampel (12), however, used both protein A and antigen-specific affinity chromatography purified anti-glucuronidase from this project in Western blots to probe for *uid*A (glucuronidase) gene expression. Although the protein A purified antibody that was supplied did not work, the antigen-specific affinity chromatography purified antibody worked well (personal communication) and gave clean blots without background cross-reactions with other *E. coli* proteins. Their success, combined with the results of the Western blot analyses, led to the belief that, although the antibody preparations seemed heterogeneous (were cross-reactive) in this project, antibody specificity was not a major problem. The reason(s) for these discrepancies between data obtained by using similar methodologies in two different laboratories cannot be explained. In retrospect, more weight on the cross-reaction data should have caused realization that the antibody preparations were not sufficient and should have been redone.

**EIA analysis**

Although the Western blot analyses proved that the antibody preparations were functional, a more substantial examination was performed. The intent of the EIA analyses was to determine if the antibody preparations were functional after solid-phase adsorption. If the antibody preparations could withstand conjugation conditions, the antibody preparations should be robust enough to be adsorbed to the latex and retain immunological activity.

The conjugated antibodies functioned well in the EIA tests (Figures 17 and 18). The

conjugated anti-glutamate decarboxylase antibodies exhibited reactivity at a 1:10,000 dilution with approximately 0.4 μg glutamate decarboxylase (Figure 17). The conjugated anti-glucuronidase antibody, however, exhibited reactivity only at a dilution of 1:100 or less with approximately 6.25 μg of glucuronidase (Figure 18). In this EIA, a 1:1000 dilution of conjugated anti-glutamate decarboxylase antibody cross-reacted with approximately 50 μg of glucuronidase enzyme. A sandwich EIA also was made with anti-glutamate decarboxylase antibody to capture glutamate decarboxylase that was subsequently detected by conjugated anti-glutamate decarboxylase antibody (data not shown). Results of the EIA analysis implied that the antibodies were functional after purification and conjugation, and should be functional after solid-phase adsorption.

EIA results reinforced the perception that the antibody preparations were active and suitable for solid-phase adsorption. Although the use of antigen-specific affinity chromatography should have eliminated the need for the establishment of antibody titers as a standardization method, further EIA work on the antibody preparations could also have been used to titer the antibody preparations and insure that they were equally reactive. This would have eliminated any antibody preparation bias that might be caused by using separate serum samples.

## SUMMARY AND CONCLUSIONS

The antibody preparations used in this study were on the basis of the methods of Kaspar (31) and Holt (21). Because their work had yielded antibody preparations that seemed to have adequate sensitivities and specificities, only minor modifications to their protocols were made. In retrospect, preparation of the antibodies should have included several more changes from the methods used by Kaspar. Foremost among the changes is the need for higher purity antigens, although this might not solve the problems encountered. The commercial enzymes, used for antibody production, probably were not as homogeneous as is needed for the production of antibodies suitable for latex agglutination tests. It is possible that antibody preparations used to sensitize latex for latex agglutination tests must be more specific than those used for other immunological tests, such as coagglutination and EIA. Whether this is true or not was not resolved in the present studies, although the results suggest the need for more specific antibody preparations for latex sensitization.

A potentially significant source of error in the preparation of the antibodies was the lack of pooling of the various antisera. Each rabbit bleed was, for the most part, stored and purified separately during the course of this project. It was believed that the use of antigen-specific affinity chromatography would compensate for differences in the purified antibodies. Pooling of each antisera, however, would have eliminated differences in antibody performance that could be attributed to variations in antibody titers that arose during the antibody production phase.

Methods, such as bulk gel electrophoresis, could be used to obtain better antigens. Although proteins are denatured during gel electrophoresis, antibody responses are not usually reduced and adequate antibody preparations are normally obtainable (19). In addition, an immunization protocol using either selective *in vitro* or *in vivo* depletion (1) could be used to produce a more specific antibody preparation. A more consistent source of good quality

antibodies might have made a significant difference in this project.

Antigen-specific affinity chromatography purification also could be used to refine antibody preparations if a relatively pure antigen composed the affinity ligand. Commercial enzyme sources, other than Sigma, were sought for their use in antigen-specific affinity chromatography purification, but none were found. Near the end of the project it was discovered that Dr. David Metzler (Department of Biochemistry and Biophysics, Iowa State University) was working on two of the enzymes: glutamate decarboxylase and tryptophanase. Although samples of the enzymes were obtained, there was not enough time to evaluate them for use as antigens. The enzymes were used for the preparation of antigen-specific affinity chromatography columns for purification of antibody preparations (used in part 3 of this dissertation).

The use of monoclonal antibodies should be considered in future attempts. Although monoclonal antibodies were considered initially, they have disadvantages that preclude their development and use. Monoclonal antibodies have a poor inherent ability to form the cross linkages necessary for agglutination assays. Although a pool of several monoclonal antibody preparations might alleviate the problem with formation of cross linking, monoclonal antibodies also are difficult to adsorb to latex (3, 4, 28, 45) and often require covalent or other chemical coupling to the latex particles. Monoclonal antibodies also require excessive time and effort for screening and production compared to the production of polyclonal antibodies. Only one monoclonal antibody to an *E. coli* enzyme has been reported (25, 26, 27, 30) but attempts to obtain the antibody were unsuccessful. Because of the successful use of polyclonal antibodies by Kaspar (31) and Holt (21), polyclonal antibodies should have been sufficient for latex agglutination tests. Even with an assay designed around monoclonal antibodies, initial antibody development probably would require purer antigens than were used in this project.

Although the antibody preparations were not prepared from homogeneous antigens and

antigen-specific affinity chromatography was not able to compensate for the lack of purity, the antibodies were considered good enough for further latex agglutination assay development. This idea was on the basis of three pieces of information. Foremost was that during the characterization of the antibodies, other experimental evidence was found that suggested that the entire problem with nonspecific agglutination was not the fault of the antibody preparations (see Part 2). In addition, because of the results of the Ouchterlony tests, Western blot controls, and EIA analyses, the reactivities of the antibody preparations were thought limited to the enzyme preparations and did not necessarily extend to other proteins. Finally, the successes by Kaspar (31) and Holt (21), and later by Feng and Lampel (personal communication), indicated that polyclonal antibodies should have sufficed.

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TABLE 1. Summary of Ouchterlony immunodiffusion results

| Antibody | Antibody purification method | Antibody titer (antigen constant) (mg/ml) | Antigen titer (antibody constant) (mg/ml) | Cross-reactivity |
|---|---|---|---|---|
| Anti-glucuronidase | Protein A | 0.0625 - 0.03125 | 0.5 - 0.0625 | none |
| | DEAE | <0.03125 | 0.5 | none |
| | Gentle method | 0.0625 | 0.5 | none |
| | AgSAC[a] | <0.03125 | 0.0625 | none |
| Anti-tryptophanase | Protein A | 0.125 | 1 - 0.5 | glucuronidase glutamate decarboxylase |
| | DEAE | not detected | not detected | none |
| | Gentle method | 0.0625 | 0.5 | none |
| | AgSAC | 0.125 | 0.25 | none |
| Anti-glutamate decarboxylase | Protein A | <0.0625 | 0.25 | tryptophanase |
| | DEAE | <0.0625 | 1.0 | tryptophanase |
| | Gentle method | <0.0625 | 0.25 | none |
| | AgSAC | <0.0625 | 0.5 | tryptophanase |
| | Kaspar's antibody | <0.0625 | 0.5 | tryptophanase |

[a]AgSAC - antigen-specific affinity chromatography

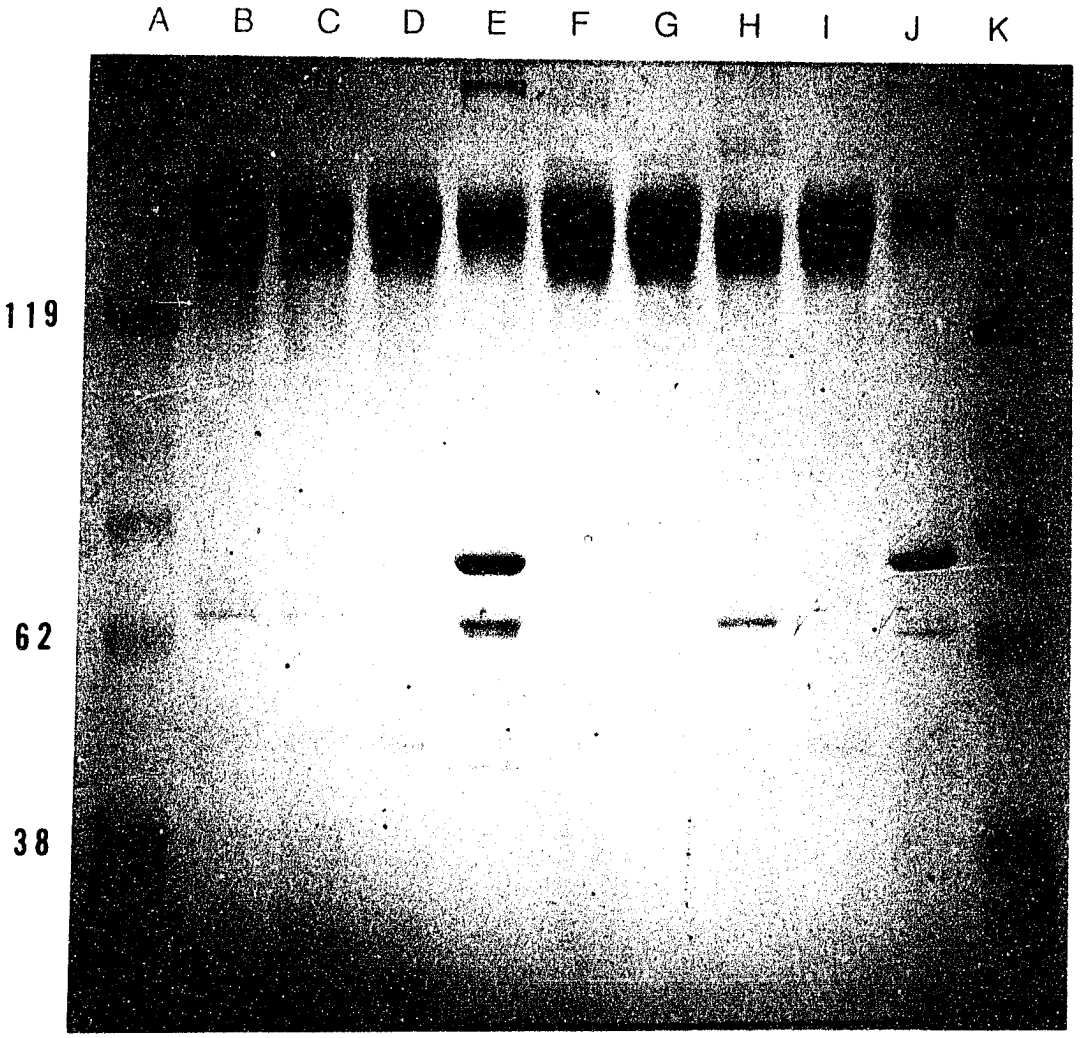
Figure 1. EIA scheme for anti-glutamate decarboxylase assay. Except column 3, columns 1 through 12 contain doubling dilutions of glutamate decarboxylase starting with 0.1 mg/ml in column 1 through 0.0000977 mg/ml in column 12. Column 3 contains BSA a negative control. Rows A and H are blank. Rows B (1:10,000), C (1:5,000), D (1:1,000), E (1:500), F (1:100), and G (1:10) contain dilutions of alkaline phosphatase conjugated anti-glutamate decarboxylase.

| | | 1 | 1:2 | BLANK | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| BLANK | A | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:10,000 | B | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:5,000 | C | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:1,000 | D | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:500 | E | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:100 | F | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:10 | G | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| BLANK | H | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |

Figure 2. EIA scheme for anti-glucuronidase assay. Except column 3, columns 1 through 12 contain doubling dilutions of glucuronidase starting with 0.1 mg/ml in column 1 through 0.0000977 mg/ml in column 12. Column 3 contains BSA a negative control. Rows A, C, and H are blank. Row B contains anti-glutamate decarboxylase. Rows D (1:10,000), E (1:1,000), F (1:100), and G (1:10) contain dilutions of alkaline phosphatase conjugated anti-glucuronidase.

| | | 1 | 1:2 | BLANK | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| BLANK | A | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| Anti-GAD | B | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| BLANK | C | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:10,000 | D | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:1,000 | E | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:100 | F | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 1:10 | G | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| BLANK | H | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |

Figure 3. Anti-tryptophanase and anti-glucuronidase native PAGE. Lanes A and K contain prestained molecular weight standards (Sigma, product number SDS-7B). Lanes B through E contain anti-tryptophanase purified by protein A, antigen-specific affinity chromatography, the gentle method, and the DEAE method, respectively. Lane F contains commercial rabbit immunoglobulin as a control. Lanes G through J contain anti-glucuronidase purified by protein A, antigen-specific affinity chromatography, the gentle method, and the DEAE methods, respectively. Approximate molecular masses are indicated along the left side.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D
E
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G
H
I
J
K
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38

Figure 4. Anti-glutamate decarboxylase native PAGE. Lanes A and G contain prestained molecular weight standards (Sigma, product number SDS-7B). Lanes B through F contain anti-glutamate decarboxylase purified by protein A, antigen-specific affinity chromatography, the gentle method, the DEAE method, and protein A from a previous investigator, respectively. Lane H contains a low molecular weight standard (Sigma, product number SDS-7B). Lane I contains commercial rabbit immunoglobulin (Sigma, product number I5006) as a control. Lane J contains bovine serum albumin (Sigma, product number A7030) as a molecular weight marker. Approximate molecular masses are indicated along the left side.

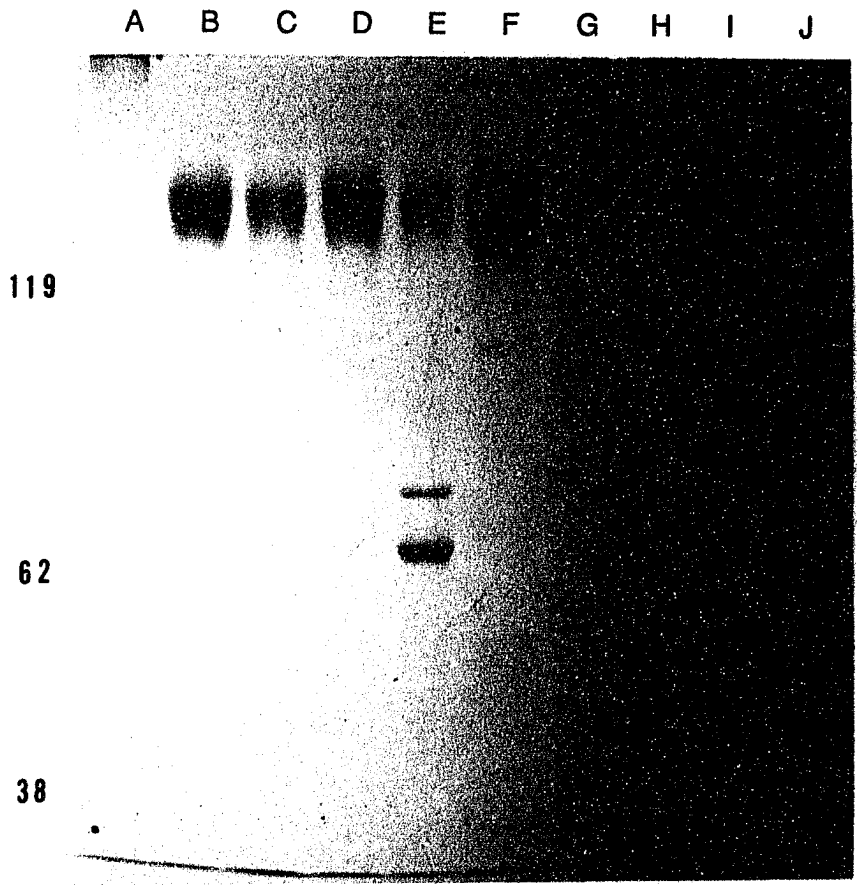
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Figure 5. Anti-tryptophanase denatured PAGE. Lane A contains normal rabbit serum. Lanes B and G contain prestained molecular weight standards (Sigma, product number SDS-7B). Lanes C through F contain anti-tryptophanase purified by protein A, antigen-specific affinity chromatography, the DEAE method, and the gentle method, respectively. Lane H contains commercial rabbit immunoglobulin (Sigma, product number I5006) as a control. Lane I contains bovine serum albumin (Sigma, product number A7030) as a molecular weight marker. Approximate molecular masses are indicated along the left side.


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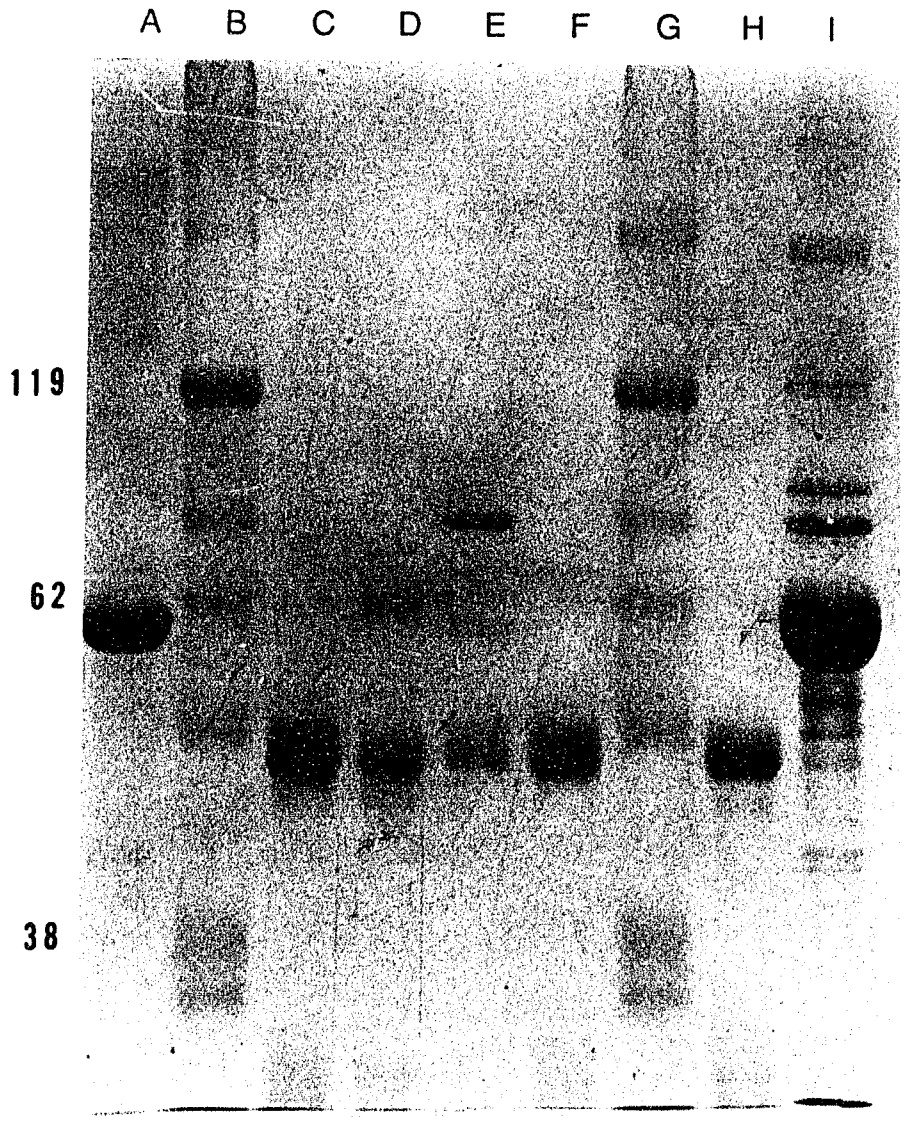
Figure 6. Anti-glucuronidase denatured PAGE. Lane A contains bovine serum albumin (Sigma, product number A7030) as a molecular weight marker. Lanes B and G contain prestained molecular weight standards (Sigma, product number SDS-7B). Lanes C through F contain anti-glucuronidase purified by protein A, antigen-specific affinity chromatography, the DEAE method, and the gentle method, respectively. Lane H contains commercial rabbit immunoglobulin (Sigma, product number I5006) as a control. Lane I contains normal rabbit serum. Approximate molecular masses are indicated along the left side.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62
38

Figure 7. Anti-glutamate decarboxylase denatured PAGE. Lanes A and G contain prestained molecular weight standards (Sigma, product number SDS-7B). Lanes B through F contain anti-glutamate decarboxylase purified by protein A, antigen-specific affinity chromatography, the gentle method, the DEAE method, and previously protein A purified antibodies, respectively. Lane H contains commercial rabbit immunoglobulin (Sigma, product number I5006) as a control. Lane I contains bovine serum albumin (Sigma, product number A7030) as a molecular weight marker. Approximate molecular masses are indicated along the left side.

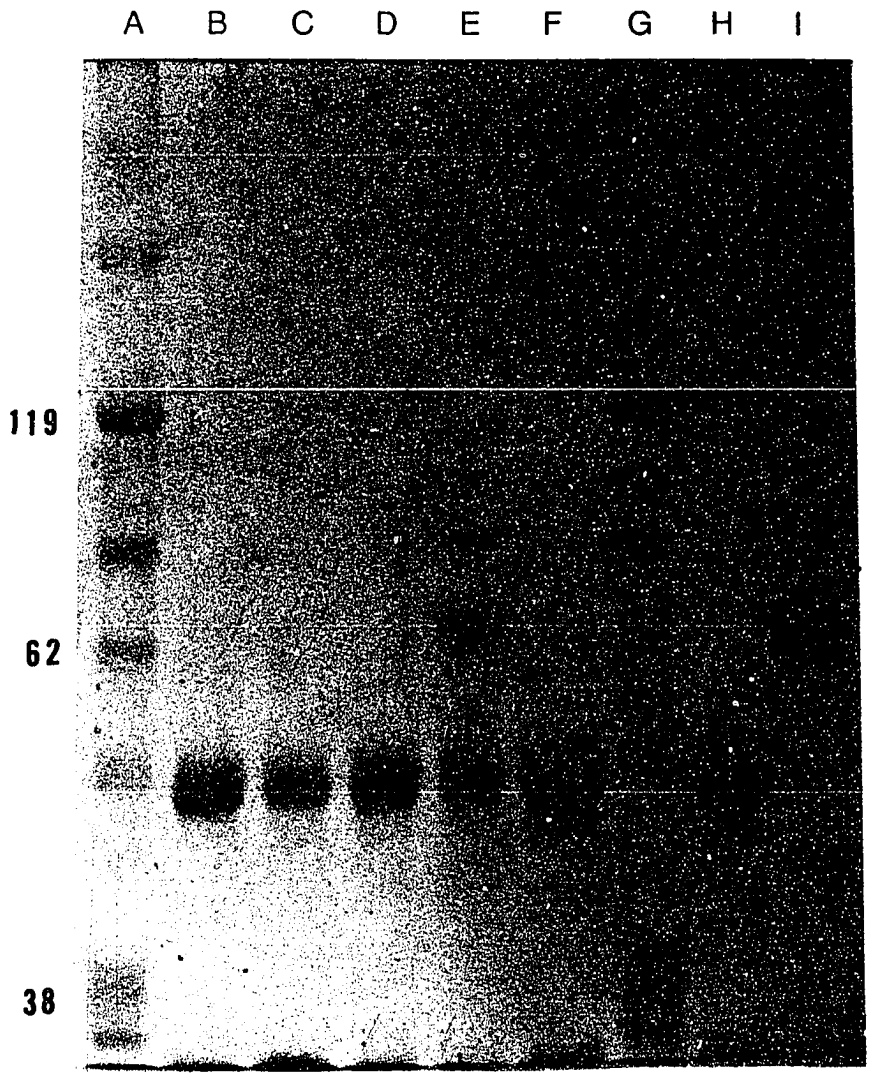

A
B
C
D
E
F
G
H
I
119
62
38

Figure 8. Anti-tryptophanase Ouchterlony.

Figure 8-A; Protein A purified anti-tryptophanase titers;

| WELL | CONTENTS |
|---|---|
| Center (E) | 2.0 mg/ml tryptophanase |
| 1 | 1.0 mg/ml anti-tryptophanase |
| 2 | 0.5 mg/ml anti-tryptophanase |
| 3 | 0.25 mg/ml anti-tryptophanase |
| 4 | 0.125 mg/ml anti-tryptophanase |
| 5 | 0.0625 mg/ml anti-tryptophanase |
| 6 | blank (buffer) |

Figure 8-B; Protein A purified anti-tryptophanase cross-reactions;

Not done.

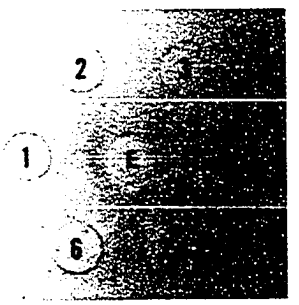
Figure 8-C; Antigen-specific affinity chromatography purified anti-tryptophanase titers;

| WELL | CONTENTS |
|---|---|
| Center (E) | 0.5 mg/ml tryptophanase |
| 1 | 1.0 mg/ml anti-tryptophanase |
| 2 | 0.5 mg/ml anti-tryptophanase |
| 3 | 0.25 mg/ml anti-tryptophanase |
| 4 | 0.125 mg/ml anti-tryptophanase |
| 5 | 0.0625 mg/ml anti-tryptophanase |
| 6 | blank (buffer) |

Figure 8-D; Antigen-specific affinity chromatography purified anti-tryptophanase cross-reactions;

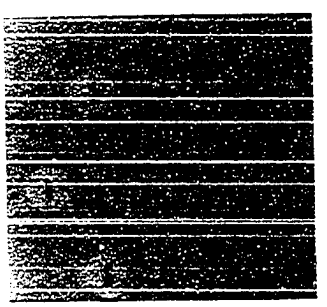
| WELL | CONTENTS |
|---|---|
| Center (E) | 1 mg/ml anti-tryptophanase |
| -1 | 1 mg/ml glucuronidase |
| 2 | 1 mg/ml tryptophanase |
| 3 | 1 mg/ml glutamate decarboxylase |
| 4 | 1 mg/ml glucuronidase |
| 5 | blank (buffer) |
| 6 | 1 mg/ml tryptophanase |

A




B

C



D

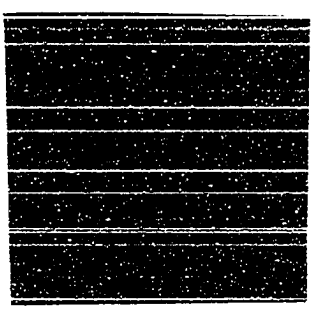


Figure 9. Anti-glutamate decarboxylase Ouchterlony.

Figure 9-A; Protein A purified anti-glutamate decarboxylase titers;

| WELL | CONTENTS |
|---|---|
| Center (E) | 1.0 mg/ml glutamate decarboxylase |
| 1 | 1.0 mg/ml anti-glutamate decarboxylase |
| 2 | 0.5 mg/ml anti-glutamate decarboxylase |
| 3 | 0.25 mg/ml anti-glutamate decarboxylase |
| 4 | 0.125 mg/ml anti-glutamate decarboxylase |
| 5 | 0.0625 mg/ml anti-glutamate decarboxylase |
| 6 | 1.0 mg/ml anti-tryptophanase |

Figure 9-B; Protein A purified anti-glutamate decarboxylase cross-reactions;

| WELL | CONTENTS |
|---|---|
| Center (E) | 1 mg/ml glucuronidase |
| 1 | 1 mg/ml anti-glucuronidase |
| 2 | 1 mg/ml anti-glutamate decarboxylase (AgSAC)[a] |
| 3 | 1 mg/ml anti-glucuronidase |
| 4 | 1 mg/ml anti-glutamate decarboxylase (proA)[b] |
| 5 | 1 mg/ml anti-glutamate decarboxylase (Kaspar) |
| 6 | 1 mg/ml anti-glutamate decarboxylase (DEAE) |

Figure 9-C; AgSAC purified anti-glutamate decarboxylase titers;

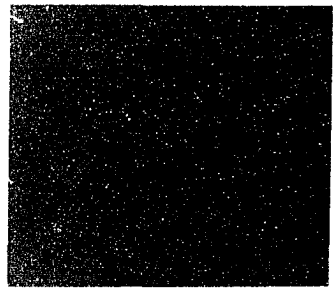
| WELL | CONTENTS |
|---|---|
| Center (E) | 1.0 mg/ml glutamate decarboxylase |
| 1 | 1.0 mg/ml anti-glutamate decarboxylase |
| 2 | 0.5 mg/ml anti-glutamate decarboxylase |
| 3 | 0.25 mg/ml anti-glutamate decarboxylase |
| 4 | 0.125 mg/ml anti-glutamate decarboxylase |
| 5 | 0.0625 mg/ml anti-glutamate decarboxylase |
| 6 | 1.0 mg/ml anti-tryptophanase |

Figure 9-D; AgSAC purified anti-glutamate decarboxylase cross-reactions;

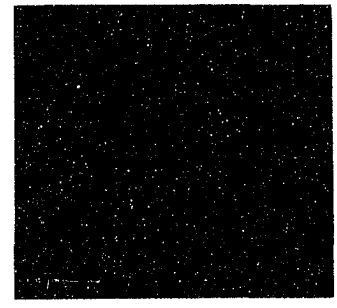
| WELL | CONTENTS |
|---|---|
| Center (E) | 25 mg/ml tryptophanase |
| 1 | 1 mg/ml anti-tryptophanase |
| 2 | 1 mg/ml anti-glutamate decarboxylase (AgSAC) |
| 3 | 1 mg/ml anti-tryptophanase |
| 4 | 1 mg/ml anti-glutamate decarboxylase (proA) |
| 5 | 1 mg/ml anti-tryptophanase |
| 6 | 1 mg/ml anti-glutamate decarboxylase (DEAE) |

[a]AgSAC = antigen-specific affinity chromatography purification
[b]proA = protein A purification

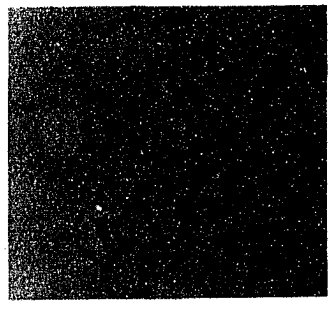
A



B



C



D

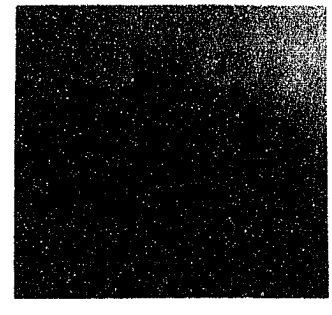


Figure 10. Anti-glucuronidase Ouchterlony.

Figure 10-A; Protein A purified anti-glucuronidase titers;

| WELL | CONTENTS |
|---|---|
| Center (E) | 1.0 mg/ml glucuronidase |
| 1 | 1.0 mg/ml anti-glucuronidase |
| 2 | 0.5 mg/ml anti-glucuronidase |
| 3 | 0.25 mg/ml anti-glucuronidase |
| 4 | 0.125 mg/ml anti-glucuronidase |
| 5 | 0.0625 mg/ml anti-glucuronidase |
| 6 | 0.03125 mg/ml anti-glucuronidase |

Figure 10-B; Protein A purified anti-glucuronidase cross-reactions;

| WELL | CONTENTS |
|---|---|
| Center (E) | 1.0 mg/ml anti-glucuronidase |
| 1 | 2.0 mg/ml tryptophanase |
| 2 | 1.0 mg/ml glucuronidase |
| 3 | 0.5 mg/ml glucuronidase |
| 4 | 1.0 mg/ml glucuronidase |
| 5 | 1.0 mg/ml glutamate decarboxylase |
| 6 | 1.0 mg/ml glucuronidase |

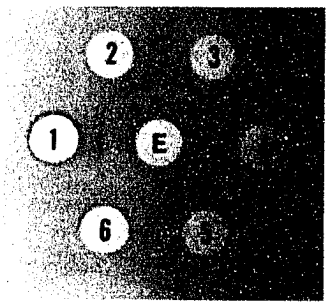
Figure 10-C; Antigen-specific affinity chromatography purified anti-glucuronidase titers;

| WELL | CONTENTS |
|---|---|
| Center (E) | 1.0 mg/ml glucuronidase |
| 1 | 1.0 mg/ml anti-glucuronidase |
| 2 | 0.5 mg/ml anti-glucuronidase |
| 3 | 0.25 mg/ml anti-glucuronidase |
| 4 | 0.125 mg/ml anti-glucuronidase |
| 5 | 0.065 mg/ml anti-glucuronidase |
| 6 | 0.03125 mg/ml anti-glucuronidase |

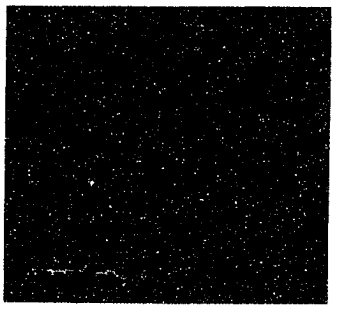
Figure 10-D; Antigen-specific affinity chromatography purified anti-glucuronidase cross-reactions;

| WELL | CONTENTS |
|---|---|
| Center (E) | 1 mg/ml anti-glucuronidase |
| 1 | 1 mg/ml tryptophanase |
| 2 | 1 mg/ml glucuronidase |
| 3 | 1 mg/ml glutamate decarboxylase |
| 4 | 1 mg/ml glucuronidase |
| 5 | blank (buffer) |
| 6 | 1 mg/ml glucuronidase |

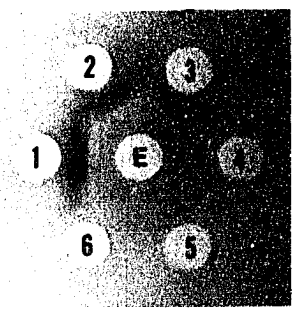
A




B



C




D

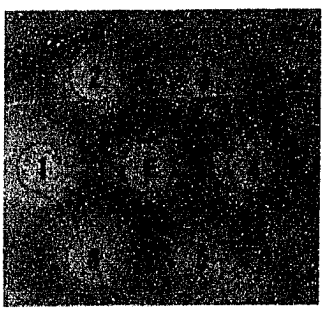


Figure 11. Denatured enzyme PAGE. Lane A contains prestained molecular welght standards (Sigma, product number SDS-7B). Lane B contains *Clostridium perfringens* glutamate decarboxylase (Sigma, product number G2251) as a control. Lane C contains *E. coli* ß-galactosidase (Sigma, product number G5635) as a control. Lane D contains tryptophanase. Lane E contains glucuronidase. Lane F contains glutamate decarboxylase. Approximate molecular masses are indicated along the left side.

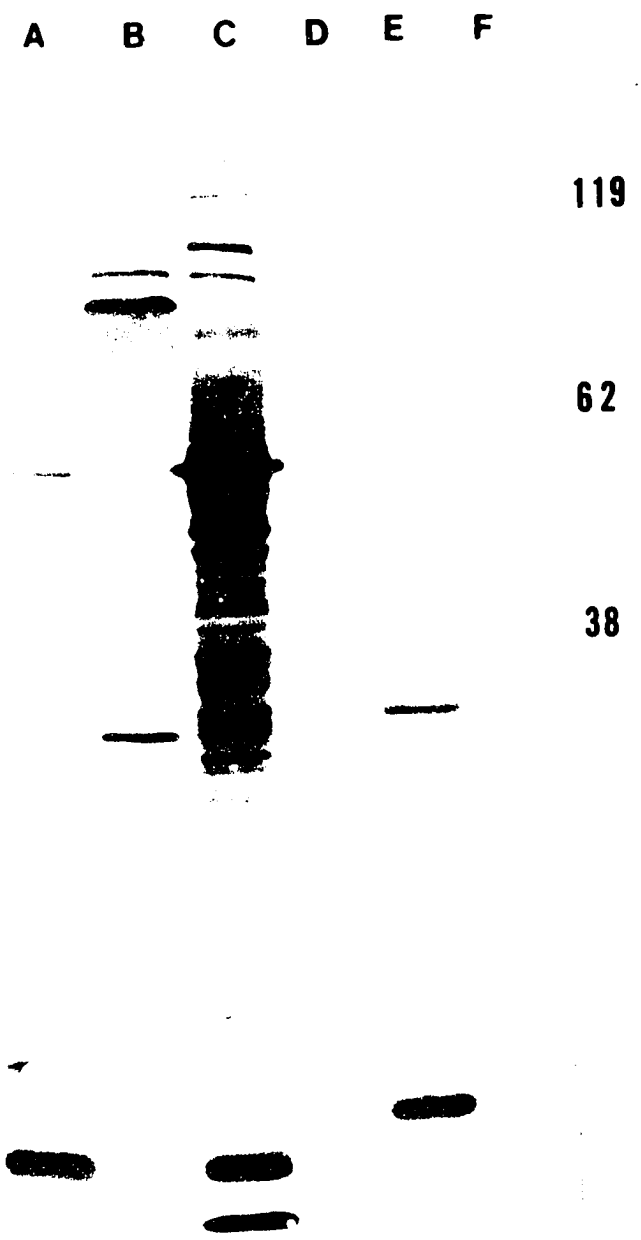

A
B
C
D
E
F
119
62
38

Figure 13. Anti-glutamate decarboxylase Western blot. Lane A contains glutamate decarboxylase. Lane B contains glucuronidase. Lane C contains tryptophanase. Lane D contains *E. coli* ß-galactosidase (Sigma, product number G5635) as a control. Lane E contains *Clostridium perfringens* glutamate decarboxylase (Sigma, product number G2251) as a control. Lane F contains prestained molecular weight standards (Sigma, product number SDS-7B; visualization of these bands is due to the prestaining and not reactivity with the primary antibody). Approximate molecular masses are indicated along the right side.

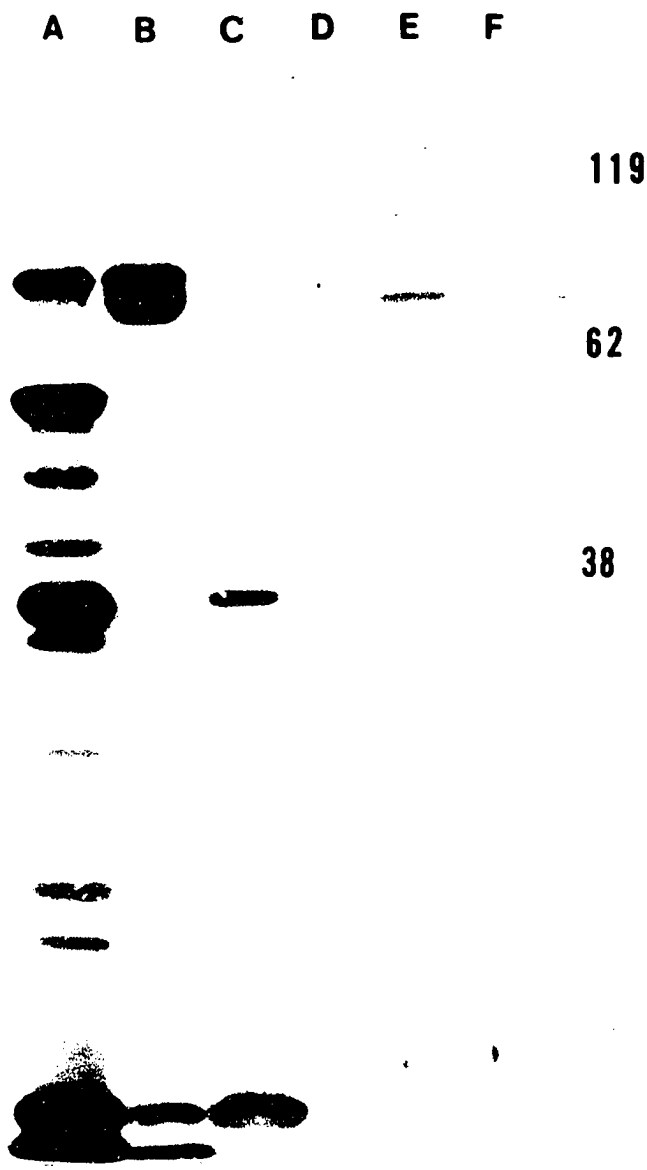

A
B
C
D
E
F
119
62
38

Figure 14. Anti-glucuronidase Western blot. Lane A contains glutamate decarboxylase. Lane B contains glucuronidase. Lane C contains tryptophanase. Lane D contains *E. coli* ß-galactosidase (Sigma, product number G5635) as a control. Lane E contains *Helix pomatia* (snail) glucuronidase (Sigma, product number G1512) as a control. Lane F contains prestained molecular weight standards (Sigma, product number SDS-7B; visualization of these bands is due to the prestaining and not reactivity with the primary antibody). Approximate molecular masses are indicated along the right side.

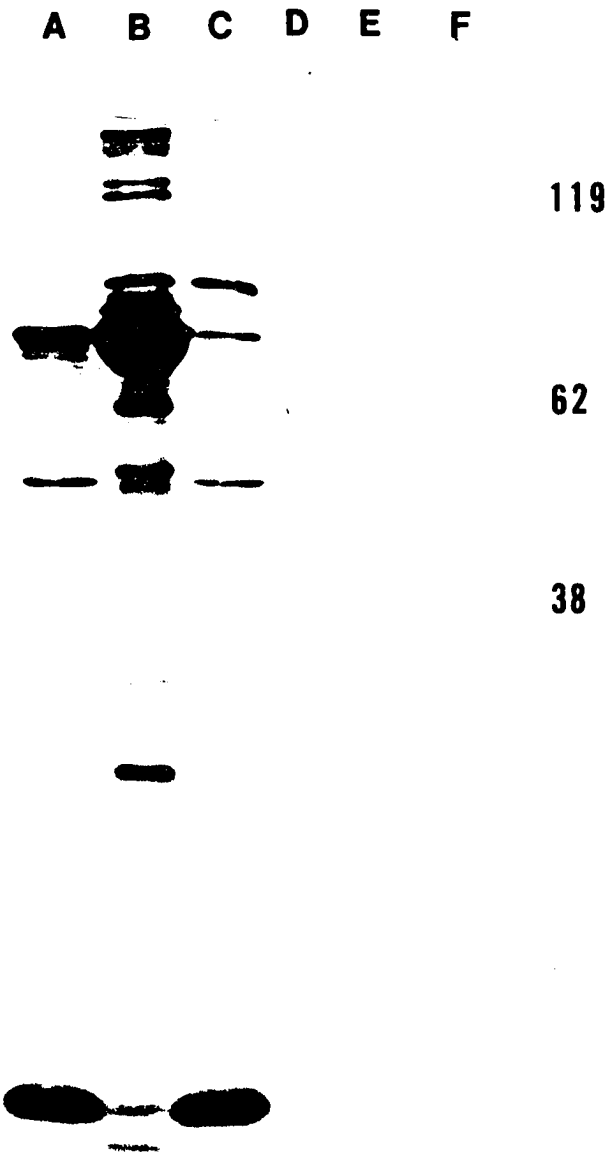

A
B
C
D
E
F
119
62
38

Figure 15. Western blot using absorbed anti-glutamate decarboxylase. Lane A contains glutamate decarboxylase. Lane B contains glucuronidase. Lane C contains tryptophanase. Lane D contains *E. coli* ß-galactosidase (Sigma, product number G5635) as a control. Lane E contains *Helix pomatia* (snail) glucuronidase (Sigma, product number G1512) as a control. Lane F contains prestained molecular weight standards (Sigma, product number SDS-7B; visualization of these bands is due to the prestaining and not reactivity with the primary antibody). Approximate molecular masses are indicated along the right side.

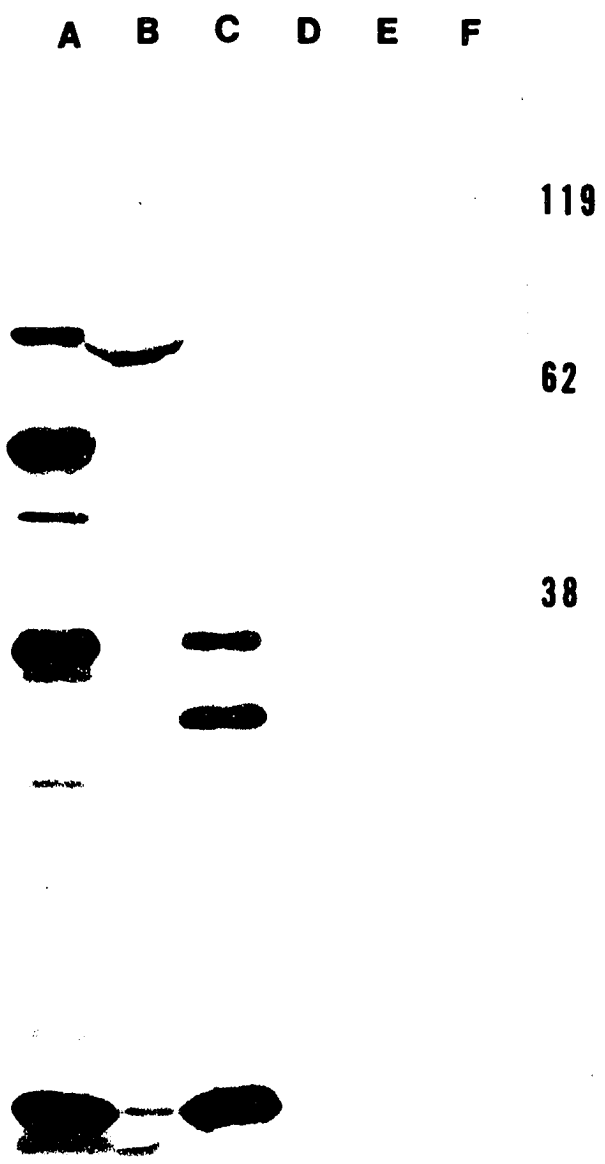

A
B
C
D
E
F
119
62
38

Figure 16. Western blot with bovine serum albumin. Lane A contains tryptophanase. Lane B contains glucuronidase. Lane C contains glutamate decarboxylase. Lanes marked X contain bovine serum albumin. An approximate molecular mass is indicated along the left side. Appropriate antigen specific affinity chromatography anti-enzyme antibodies were allowed to react with each blot.

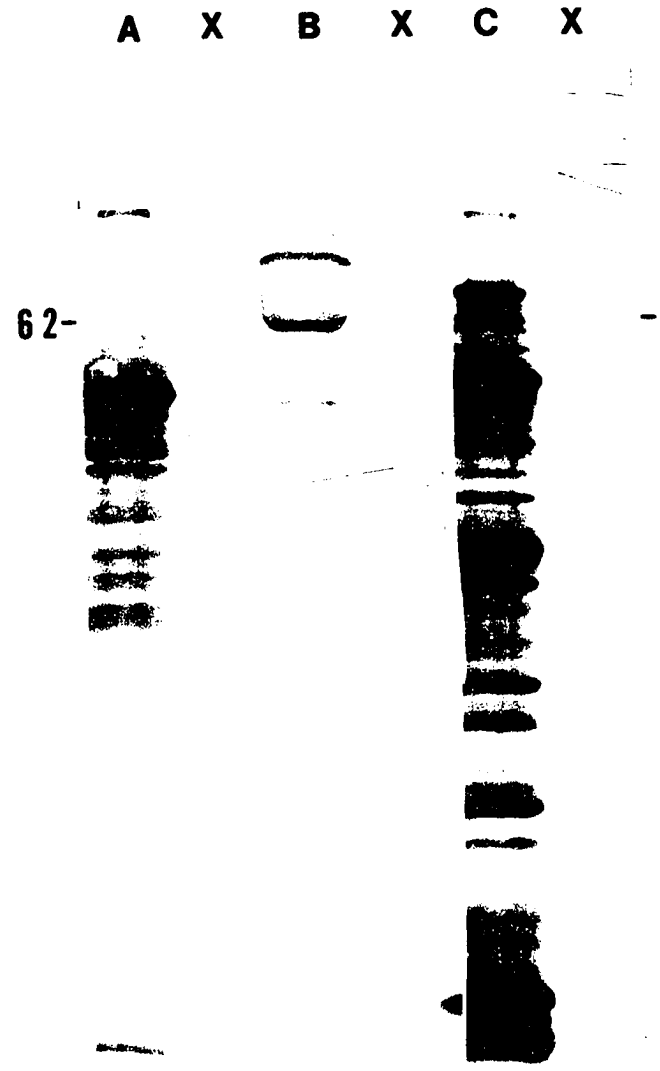

A X B X C X
62-

Figure 17. Anti-glutamate decarboxylase EIA. Except column 3, columns 1 through 12 contain doubling dilutions of glutamate decarboxylase starting with 0.1 mg/ml in column 1 through 0.0000977 mg/ml in column 12. Column 3 contains BSA a negative control. Rows A and H are blank. Rows B (1:10,000), C (1:5,000), D (1:1,000), E (1:500), F (1:100), and G (1:10) contain dilutions of alkaline phosphatase conjugated anti-glutamate decarboxylase. Conjugated anti-glutamate decarboxylase antibodies exhibited reactivity at a 1:10,000 dilution with approximately 0.4 $\mu$g glutamate decarboxylase.

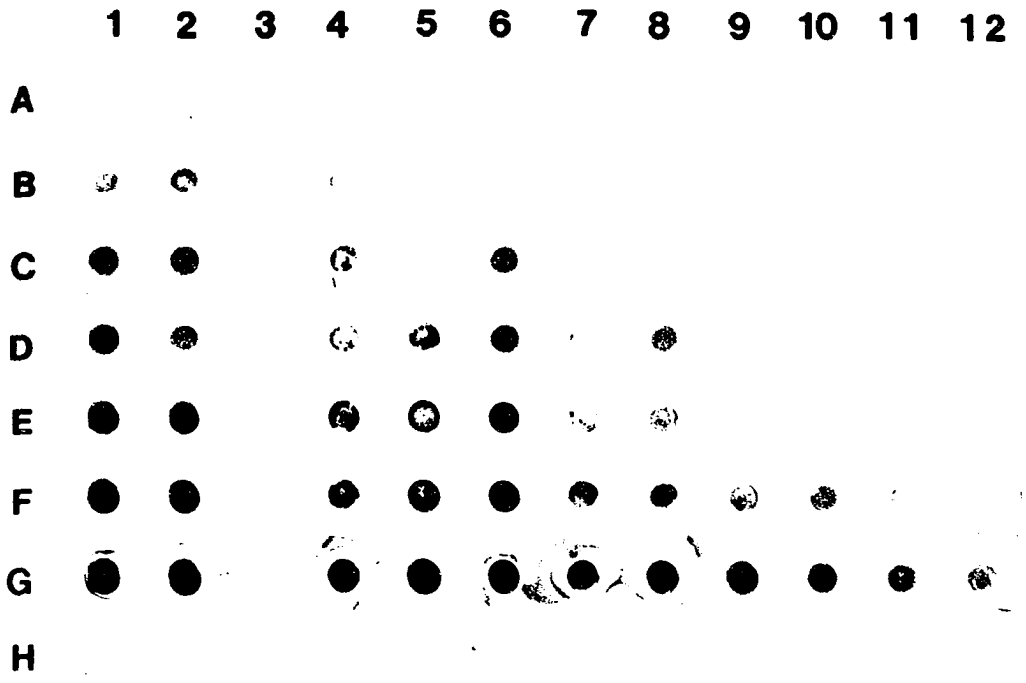
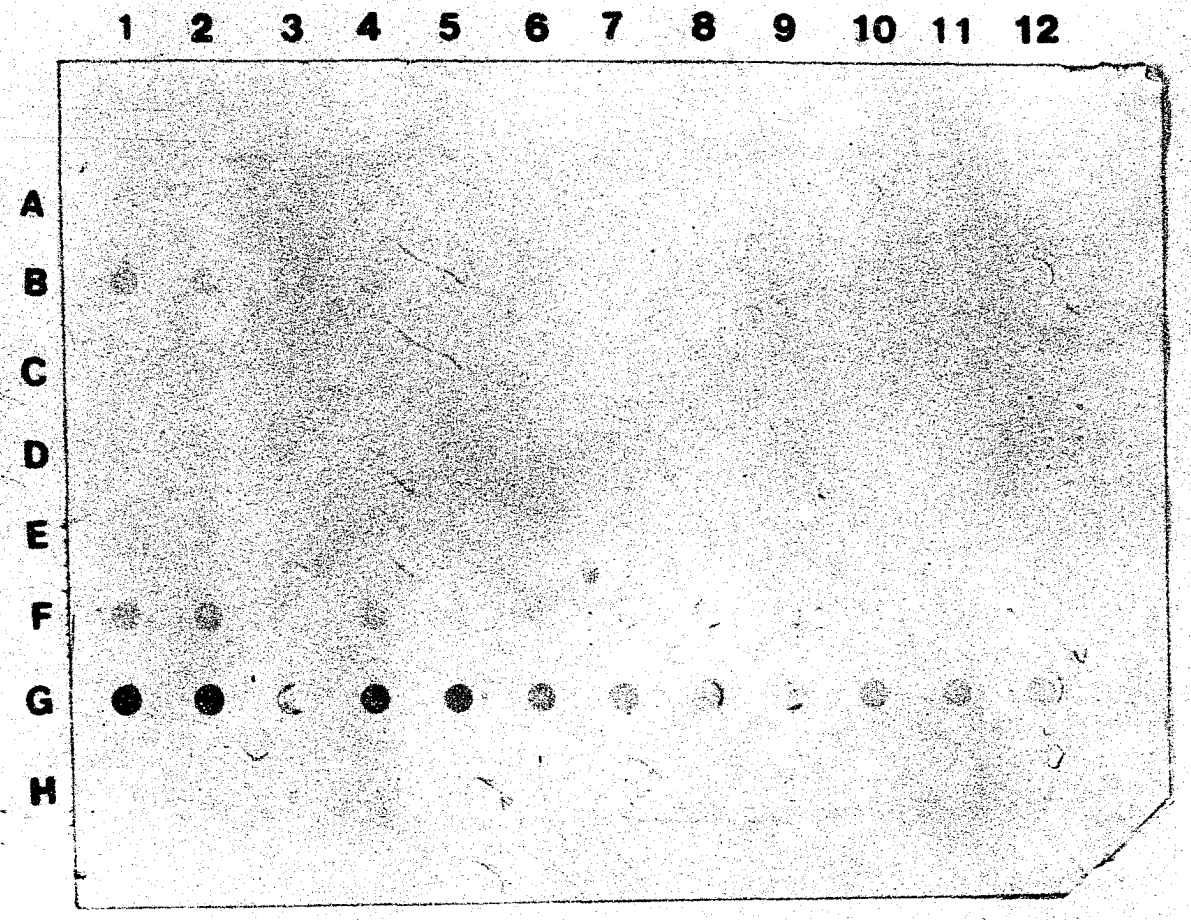

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Figure 18. Anti-glucuronidase EIA. Except column 3, columns 1 through 12 contain doubling dilutions of glucuronidase starting with 0.1 mg/ml in column 1 through 0.0000977 mg/ml in column 12. Column 3 contains BSA a negative control. Rows A, C, and H are blank. Row B contains 1:1000 dilution of conjugated anti-glutamate decarboxylase. Rows D (1:10,000), E (1:1,000), F (1:100), and G (1:10) contain dilutions of alkaline phosphatase conjugated anti-glucuronidase. Conjugated anti-glucuronidase at a 1:10 dilution reacted with as little as 97 ng of commercial enzyme preparation. A 1:100 dilution of conjugated anti-glucuronidase, however, detected only 6.25 to 12.5 $\mu$g of commercial enzyme preparation. The conjugated anti-glucuronidase did cross-react with 50 $\mu$g of commercial glucuronidase preparation.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

## PART 3. LATEX PREPARATION FOR SENSITIZATION WITH IMMUNOGLOBULINS AND ADSORPTION OF IMMUNOGLOBULINS

## INTRODUCTION

Development of latex agglutination assays require that antibodies be functionally adsorbed to latex particles. Several protocols for the adsorption of immunoglobulins to latex were examined, but the assays failed to function satisfactorily because of nonspecific agglutination. An investigation into the causes of the nonspecific agglutination was begun, especially as it pertains to the adsorption of immunoglobulins on the latex. Adsorption of protein on the latex was measured under several different conditions of buffer, protein concentration, and brand of latex. In addition, the effects of anti-enzyme and other immunoglobulins on sensitized latex were studied in attempts to determine the conditions under which the sensitized latex started to undergo nonspecific agglutination.

# MATERIALS AND METHODS

## Common Procedures Used in this Project

### *Cleaning Latex for Use*

Latex particles were cleaned before protein coating by either centrifugation washing or dialysis. For centrifugation washing, enough latex needed for each experiment was added to 1.5 or 2.0 ml microcentrifuge tube. Enough 1 M KCl was added to bring the total volume to either 1.25 or 1.8 ml, depending on the microcentrifuge tube being used. The latex suspension was mixed several times by inversion before being sedimented by centrifugation for 5 to 15 min at 13,000 rpm (approximately 11,300 × *g*) in a microcentrifuge. Liquid was aspirated from the microcentrifuge tube without disturbing the latex pellet. An appropriate volume of sterile filtered buffer was added to each tube and the latex was suspended by either vortex mixing or by ultrasonication. Each washing with buffer was performed three times before the latex was suspended at a known latex concentration in buffer.

For dialysis cleaning, enough latex needed for each experiment plus 10 mg was diluted to 2%, put into a 15 × 1.6 cm dialysis bag (molecular weight cutoff 12-14,000; SpectraPor, product number 08-667B, Fisher Scientific, Pittsburgh, PA) and placed in approximately one liter of 1 M KCl. After dialysis overnight at approximately 4°C without agitation, the KCl was replaced with Tris/azide buffer (0.03 M Tris, pH 7.4, 0.05% sodium azide) and dialysis was continued for another 24 h. The Tris/azide buffer was replaced with fresh buffer and the dialysis continued for another 24 h before the latex was used.

### *Latex Bound Protein Determinations*

The amount of protein bound to the latex particles was determined by the method suggested by Seradyn Inc. (Indianapolis, IN). Bicinchonic acid (BCA) reagent (20, 21; Pierce, catalog number 23225) was mixed according to the manufacturer's instructions. A seven-point

standard curve was prepared from horse IgG (Pierce, catalog number 23211G) diluted from 1.2 to 0.1 mg/ml. Six controls were included to monitor test performance; these consisted of a gamma globulin protein standard (Bio-Rad Laboratories, product number 500-0001) in two-fold dilutions from an initial concentration of 1.44 mg/ml. A 0.25-ml portion of each protein standard, control, four blanks, and latex samples (normally at 1%, or 0.25 mg of latex) was added to 12 × 75 mm test tubes. BCA reagent (2.5 ml) was added to each tube, mixed, and incubated at 60°C for 30 min in a water bath with gentle agitation. After incubation, the tubes were cooled to room temperature in chilled water. Solutions of standards, controls, and blanks were transferred to disposable cuvettes. Then the latex suspensions were aspirated into a 3-ml disposable syringe and passed through a filter (Millex-GS, 0.22-$\mu$m, catalog #SLGS025OS, Millipore Corp., Bedford, MA) into the disposable spectrophotometer cuvettes. The absorbance was read at 562 nm, using a reagent blank, on a Uvikon 930 spectrophotometer (Tegimenta, Switzerland). Readings were completed within 45 min after incubation to prevent continued reagent drift. A standard curve was constructed; determinations of unknown values were calculated from the standard curve using geometric and linear regression determinations. Calculation of $\mu$g protein per mg latex was then used to standardize values. The BCA reaction gives a nonlinear standard curve, so dilutions of latex suspensions were sometimes used to insure that absorbance values were within standard curve limits.

***Performance of Agglutination Assays***

Agglutination assays were performed by separately depositing 15 $\mu$l of a 1% latex suspension and 15 $\mu$l of antigen-containing solution on a glass slide circumscribed with a raised ring. The latex suspension and antigen solution were mixed until homogeneous by using a disposable plastic applicator (Despenstirs, catalog #8728-10, Becton-Dickinson, Cockysville, MD). The glass slide was incubated at room temperature on a rotator (Junior Orbital Shaker, model

3520/3522, Lab Line Instruments, Melrose Park, IL) rotating at approximately 100 rpm. A cover was used on the shaker to reduce evaporation.

Reactions were read both macroscopically and with the aid of an illuminated magnifying lens. A positive reaction was graded on a scale of ± to ++++ on the basis of degree of agglutination present. A ± reaction appears negative macroscopicaily but some agglutinated particles were observed when the preparation is examined with a magnifying lens. A + reaction has a grainy appearance macroscopically on the basis of only a few particles participating in an agglutination reaction. A ++ reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles. A +++ reaction has several agglutinated particles of a moderate size with a relatively clear background. A ++++ reaction has one or more agglutinated particle clumps with no macroscopically free particles.

**Effects of Saline on Unsensitized Latex**

Eight glycine buffered saline solutions (7.3 g/L glycine, pH 8.2) were prepared with sodium chloride concentrations ranging from 10 g/L to 0.078 g/L. Ten milligrams of blue dyed 0.81-$\mu$m latex (Bangs Laboratories Inc., Indianapolis IN) were cleaned by centrifugation washing before final suspension. The latex suspensions were observed at 10 and 30 min and 1, 2, 3, and approximately 18 h for flocculation of the latex suspension.

**Sensitization of Latex to Determine of the Effects of Different Brands of Latex and Different Buffers on Protein Adsorption**

In general, 0.5 ml of 1% sensitized latex was prepared in each of four separate experiments. In each experiment, a different 0.8-$\mu$m diameter latex from IDC (Interfaciai Dynamics Corporation, Portland, OR), Seradyn (Seradyn inc., Indianapolis, IN), Polysciences (Polysciences, Warrington, PA), or Bangs (Bangs Laboratories, Indianapolis IN) was used. Antibody sensitization of the latex was performed at antibody concentrations of 0.36, 0.18, 0.09, and 0.045 mg/ml.

Buffers used consisted of Tris/azide (0.03 M Tris, pH 7.4, 0.05% sodium azide), borate (0.34 M boric acid, pH 8.0, with 0.1% BSA and 0.05% sodium azide), HEPES (0.02 M, pH 7.5), glycine buffered saline (7.3 g/L glycine, 10 g/L NaCl, pH8.2), and phosphate buffered saline (0.01 M $NaH_2PO_4 \bullet H_2O$, 0.155 M NaCl, pH 7.2). The latex particles for these studies were cleaned by centrifugation washing. One miliitliter of different concentrations of antigen specific affinity chromatography purified anti-glucuronidase antibody solution was added to each appropriately labeled 2.0-ml centrifuge tube. Ten milligrams of washed latex were added to each tube and the volume was brought to 1.5 ml (total volume) with buffer. The tubes were mixed by inversion several times and then the latex/antibody suspensions were incubated at room temperature (approximately 27°C) overnight on a platform rocker (Thermolyne Speci-Mix, model M26125). After incubation, the latex was pelleted by centrifugation and washed once in an appropriate buffer solution before suspending it to 0.5 ml (1% (w/v) latex) with appropriate buffer. Latex bound proteins were determined on a sample of the latex suspension (see Latex Bound Protein Determinations). One ml of 1 mg/ml bovine serum albumin in the appropriate buffer was added. The latex suspensions were again incubated at room temperature (approximately 27°C) for approximately 4 h on a platform rocker (Thermolyne Speci-Mix, model M26125). After the second incubation, the latex suspensions were pelleted by centrifugation, washed twice in the appropriate buffer, and suspended to a volume of 0.5 ml with the appropriate buffer containing 1 mg/ml BSA and 0.1% thimerosal. The antibody sensitized latex was stored at 4°C.

**Sensitization of Latex to Determine Maximal Protein Binding**

In three separate experiments, 0.5 ml of 1% sensitized latex was prepared. Enough yellow dyed polystyrene latex of 0.8-$\mu$m diameter (Bangs Laboratories) was cleaned by centrifugation washing. The latex was sensitized by using antibody concentrations of 6, 3, 2, 1, and 0.5 mg/ml in Tris/azide buffer (0.03 M Tris, 0.05% sodium azide, pH 7.4). One ml of protein A purified anti-

glucuronidase antibody solution was added to an appropriately labeled 2.0-ml centrifuge tube. Ten milligrams of washed latex were added, the volume was increased to 1.5 ml total volume with buffer, and the tubes were mixed by inversion several times. The latex/antibody suspensions were incubated at 37°C overnight on a platform rocker (Thermolyne Speci-Mix, model M26125), pelleted by centrifugation, washed once with Tris/azide buffer and suspended to a volume of 0.5 ml with buffer. Latex bound proteins were determined on a sample of the latex suspension (see Latex Bound Protein Determinations). One ml of 1 mg/ml bovine serum albumin in buffer was added to block unreacted sites on the latex and the latex suspensions were incubated at room temperature (approximately 27°C) overnight on a platform rocker. After the second incubation, the latex suspensions were again pelleted by centrifugation, washed twice in buffer, and suspended to a volume of 0.5 ml (1% (w/v) latex) with buffer containing 1 mg/ml BSA and 0.1% thimerosal. The sensitized latex was stored at 4°C.

**Differences in Non-specific Agglutination of Sensitized Latex Resulting from Immunoglobulins**

***Sensitization of latex to determine the effects of antibody purification methods on nonspecific agglutination***

The effects of antibody purification methods on the enzyme-latex agglutination assay were examined. Antigen-specific affinity chromatography purified, DEAE column purified, protein A purified, and, in the case of anti-glutamate decarboxylase, gentle purified antibodies were used to sensitize the latex. Blue and red dyed polystyrene latex particles, 0.8 $\mu$m diameter (Bangs Laboratories) were cleaned by centrifugation washing as previously described. Ten milligrams of latex (blue dyed latex for anti-glutamate decarboxylase and red dyed latex for anti-tryptophanase) were added to antibody solutions and the suspensions incubated at 37°C for 2 h. In addition, the latex was coated with BSA (0.047) and carried through the sensitization as a negative control. After sedimentation of the latex by centrifugation at 13,000 rpm (approximately 11,300 $\times$ *g*) in a

microcentrifuge, the antibody solutions were removed and filtered through a 0.22- or 0.45-$\mu$m syringe filter. The recovered antibody protein concentration was estimated by a spectrophotometric method, using $A_{280}$ values and an extinction coefficient of 1.4 (3, 6). Two milliliters of buffer containing 0.1% BSA and 0.05% polyvinylpyrrolidone (molecular weight 40,000; Sigma, product number PVP-40) were added and the suspension was allowed to incubate at room temperature overnight before use.

The latex was tested against 0.1, 0.01, and 0.001 mg/ml of appropriate enzyme solution. (Appropriate refers to using an enzyme as an antigen to which the latex was sensitized with anti-enzyme antibody preparations). In addition, controls consisting of distilled water, 1 M $KH_2PO_4$ buffer, 0.1 mg/ml glucuronidase (Sigma, product number G7896), 0.1 mg/ml glutamate decarboxylase (Sigma, product number G3757), and 0.1 mg/ml apotryptophanase (Sigma, product number A6007) were used.

***Sensitization of latex to determine nonspecific agglutination of commercial anti-glucuronidase***

Anti-glucuronidase solutions containing 0.04, 0.05, 0.06, and 0.07 mg/ml of protein were prepared from four different sources: antigen-specific affinity chromatography purified, protein A purified, Molecular Probes (Molecular Probes, Inc., product number A-5790), and 5′3′ (5Prime$^-$ 3Prime Inc., product number 5307-246468). Protein estimates were performed by using $A_{280}$ values and an extinction coefficient of 1.4 (3, 6). Ten milligrams of washed 0.81-$\mu$m yellow dyed polystyrene (Bangs Laboratories) were added to 1.75 ml of each antibody containing solution (total volume 2 ml) and mixed by inversion. The latex suspensions were incubated for 1 h at 37°C with intermittent mixing by inversion followed by 1 h stationary. Tris/azide buffer (0.25 ml) containing 4 mg/ml BSA was added and allowed to incubate with the latex suspension stationary, overnight, at 4°C. The latex suspension was washed three times before 200 $\mu$l of 0.8% latex was suspended in assay buffer. The assay buffers consisted of glycine buffer (0.03 M, pH 8.2, with 0.1%

BSA and 0.05% sodium azide), phosphate buffer (0.1 M $NaH_2PO_4$-$H_2O$, 0.155 M NaCl, pH 6.6, with 0.1% BSA and 0.05% sodium azide), borate buffer (0.34 M boric acid, pH 8.0, with 0.1% BSA and 0.05% sodium azide), and Tris/azide buffer (0.03 M, pH 7.4, with 0.1% BSA and 0.05% sodium azide). Saline was prepared with concentrations ranging from 0.43, 0.26, 0.17, 0.09, 0.04, and 0.02 M.

***Sensitization of latex with irrelevant immunoglobulins to determine immunoglobulin-induced nonspecific agglutination***

Anti-glucuronidase from Molecular Probes (Molecular Probes, Inc., product number A-5790), horse IgG (Pierce, catalog number 31126G), mouse IgG (Pierce, catalog number 31204G), or rabbit IgG (Sigma, product number I5006) solutions containing 1 and 0.01 mg/ml of protein were prepared. Protein A purified anti-glucuronidase and normal rabbit serum (1:1) solutions containing 1 and 0.1 mg/ml of protein were prepared. Protein estimates were performed by using $A_{280}$ values and an extinction coefficient of 1.4 (3, 6). Ten milligrams of washed 0.81-$\mu$m polystyrene latex (Seradyn) were added to 1.75 ml of each antibody containing solution (total volume 2 ml) and mixed by inversion. The latex suspensions were incubated for 2 h at 37°C on a platform rocker (Thermolyne Speci-Mix, model M26125). The latex suspensions were pelleted by centrifugation, washed once with Tris/azide buffer and suspended to a volume of 0.5 ml with buffer. Latex bound proteins were determined on a sample of the latex suspension (see Latex Bound Protein Determinations). One ml of 10 mg/ml bovine serum albumin in buffer was added to block unreacted sites on the latex and the latex suspensions were incubated at 37°C overnight on a platform rocker. After the second incubation, the latex suspensions were again pelleted by centrifugation, washed twice in Tris/azide buffer, and suspended to a volume of 0.5 ml (1% (w/v) latex) with TPG buffer (7.8 g/L $NaH_2PO_4 \cdot 2H_2O$, 1 g/L NaCl, 2 g/L gelatin, pH 6.6). The sensitized latex was stored at 4°C.

## RESULTS AND DISCUSSION

### Effects of Saline on Unsensitized Latex

During the work with immunoglobulin adsorption to latex, it was noticed that in some buffers the latex would settle out of suspension. Originally, this was thought a result of the adsorption of immunoglobulins to the latex. Later, it was reexamined to determine if there was a correlation between the degree of settling and the degree of nonspecific agglutination obtained with sensitized latex. (There was a positive correlation; data not shown). Studies of unsensitized latex in saline solutions also were completed.

Suspensions of latex were all evenly dispersed after 10 min (Table 1). After 30 min, however, flocculation could be observed at the greatest saline concentration (10 g/L) and flocculation increased over the next 2 h. In the 5 g/L concentration of saline, flocculation started after about 2 h and increased to 1+ by 3 h. In the 2.5 g/L concentration of saline, a slight flocculation developed only after incubation overnight. Concentrations of saline less than 2.5 g/L did not cause flocculation of the latex over the period of observation.

The results demonstrated that the flocculation of latex in suspension was caused, in part, by the concentration of saline in the suspending buffer. Saline evidently decreases the zeta potential (the innate charges that surround particulate solid-phases; 14, 25) that keeps the latex particles repelled from each other and at saline concentrations above about 2.5 g/L, flocculation starts to occur. The significance of this observation was that many buffers recommended for use in protein coating of latex contains concentrations of saline that are greater than 2.5 g/L (NaCl at 9 to 10 g/L is normal in these buffers). This could be detrimental to efficient protein absorption on latex. If latex particles start flocculating before maximum quantities of protein are absorbed to the particles, the protein could cross-link the particles and cause tightly bound latex aggregates.

The results of this study suggested that a change from glycine buffered saline to Tris/azide

buffer (no saline) would be beneficial. In addition, the results of several other studies demonstrated that Tris/azide was a suitable buffer system for protein adsorption. The latex bound protein studies (data follow later) suggested that Tris/azide performed well for adsorption of immunoglobulins, Tris/azide resulted in less background color in the latex bound protein determinations, and subjective observations of flocculation during immunoglobulin adsorption with Tris/azide buffer indicated that spontaneous flocculation of the latex particle was significantly less. With this information, Tris/azide became the most commonly used adsorption buffer for immunoglobulin adsorption to the latex particles.

## Protein Adsorption to Latex

### *Comparison of latex brands*

To insure that immunoglobulins were adsorbing to the latex particle, a series of experiments was accomplished specifically looking at immunoglobulin adsorption. With the use of BCA reagent, the quantity of protein adsorbed to the latex could be directly measured rather than calculated from an analysis of residual protein in the adsorption solutions.

Figure 1 shows a comparison of protein binding by four brands of latex particles (all 0.8 $\mu$m diameter polystyrene). Two of the four brands of latex bound more protein than was bound by the other two brands of latex. In one instance (Polysciences), the results exceeded the standard error of the means for two other latexes (Bangs and Seradyn; error bars not shown for the difference in the four replicates). In the other instance (IDC), although not statistically significant (the error bars overlapped), the same trend was noted. Thus, there were differences among the brands of latex in their ability to bind rabbit immunoglobulins. The results of the studies on latex brands established that Polysciences latex, the original brand used, was not the best latex for adsorption of immunoglobulins. On the basis of these results, sensitization of latex particles for the latex

agglutination assay was subsequently restricted to particles from either Seradyn or Bangs Laboratories.

Analysis of the results corroborates Finkelstein and Yang's (5) demonstration of differences in brands of latex. This work was different from Finkelstein and Yang's, however, because the data were quantitatively based on the amount of protein bound to the latex rather than on the subjective evaluation of the sensitivity of the assay. The study did not involve evaluating different lots of latex from the same manufacturer. A more thorough study of differences in immunoglobulin binding to several lots of latex from each manufacturer might have added additional information. Although an attempt was made to use one lot number of latexes for the entire project, when the original samples of latex had been consumed, other lot numbers of latex were used without further testing of the adsorption characteristics. Redoing the protein adsorption studies should have been accomplished to insure that the new latex lots performed as well as the original material.

Although the purpose of these studies was to confirm that immunoglobulins adsorbed to the latex particles, changes in the total volume, percent solids, temperatures of adsorption, mechanical agitation, and other parameters, could influence protein adsorption. Further studies on the effects of these parameters could have been accomplished to insure that the optimal conditions were obtained. After insuring that adequate immunoglobulin adsorption occurred in this study, however, a decision was made to proceed with other areas of investigation so that the project was not side tracked into studies of protein adsorption to latexes. Such studies would have been performed if satisfactory latex agglutination assays had been developed to optimize the assays.

***Comparison of binding buffers***

As part of the investigation of immunoglobulin adsorption to different brands of latex, the effect of buffers on immunoglobulin adsorption also was tested. Protocols on protein adsorption to latex from different sources included the use of different buffers. Although information exists on the

effects of different buffers on protein adsorption (7, 23), the findings were substantiated for application in this project.

Figure 2 shows the quantities of protein bound by different brands of latexes sensitized in the presence of different buffers. In general, neither the buffer type nor the pH significantly affected the amount of immunoglobulins adsorbed to the latex. This is consistent with previously published work (7, 23) that suggest that protein adsorption is independent of pH.

Although a definitive buffer was not identified as superior for the adsorption of immunoglobulins, the information gained in the study of different buffers was useful. By knowing that there were no differences in the effects of buffers or pH on the adsorption of immunoglobulins to latex, unconstrained substitution of adsorption buffers could be made. This was done when Tris/azide was substituted for glycine buffered saline after the effects of saline on unsensitized latex became known.

**Determination of Maximum Protein Binding**

After the determination of immunoglobulin adsorption, differences among brands of latex adsorbed on the latex particle was determined. Until this time, the amount of immunoglobulin used had been estimated by extrapolation of protein-adsorption results on other types of polystyrene by other investigators (2).

Figure 3 shows the relationship between the quantity of protein used and the quantity bound to latex. The results approximate a curve with maximal protein binding at approximately 1 mg/ml of protein applied. The curve follows the formula:

$$Y = \frac{(4.1624 + 366.18X)}{(1 + 3.43X)}$$

The results, illustrating a saturation curve for protein binding, are consistent with other investigators' work (4, 10, 17, 18, 22). This knowledge was useful in deciding how much protein should be used

to saturate latex preparations for other experiments. Some sources (2, 16) suggested that less than saturation of the latex with antibodies is desirable because of prozone effects. (The prozone is a phenomenon exhibited in which agglutination occurs within a range of equivalence of antibodies and antigens; outside which, optimal agglutination is hindered by a reactant excess). The results from studies on immunoglobulin-sensitized latex suggested that nonspecific agglutination might be directly influenced by the amount of immunoglobulin bound to the latex (data follow). By working within the parameters of the saturation curve, a better sensitized latex reagent should be achieved.

Extrapolation of the other polystyrene results to latex provided an incorrect estimate for the adsorption of immunoglobulins on latex, and the data in this study were more consistent with subsequent information (17). The adsorption data were used throughout the remainder of the project to determine the degree of immunoglobulin saturation on the latex.

**Differences in Non-specific Agglutination of Sensitized Latex Resulting from Immunoglobulins**

***Effects of antibody purification methods on nonspecific agglutination***

Because Kaspar (8) had used polyclonal antibodies made in-house that evidently performed well in his assays, an original consideration when the present latex agglutination tests were not performing as expected, was to look at the antibody purification method. Kaspar had used protein A purified antibodies for coaggiutination and enzyme-capture assays (8). In the present study, antigen-specific affinity chromatography purified antibody preparations were being used (Part 1). It is known that the harsh conditions of antibody elution from antigen-specific affinity chromatography columns (pH 2.8) can cause some physical changes in immunoglobulin structure (11). Because of differences in purification methods, it was reasoned that a change in the purification method could result in antibodies not as prone to nonspecific agglutination. Changes to protein A, DEAE, and gentle antibody purification methods (see part 1 of this dissertation) were examined to determine

which method(s) might yield antibody preparations not prone to nonspecific reactions when used to sensitize latex.

Antibodies purified by protein A, antigen-specific affinity chromatography, DEAE, and gentle elution from protein A, were all predisposed to nonspecific agglutination (Table 2). It was noted in this set of experiments, however, that nonspecific agglutination appeared only when immunoglobulins were adsorbed to the latex; BSA-coated latexes were nonreactive in all the test solutions.

Although there were slight differences in the reactivities of antibodies prepared by different methods, the differences were not considered significant. On the basis of the potential for reduced cross-reactivity of the antibodies, antigen-specific affinity chromatography purified antibodies continued to be used most often for further studies. Owing to the low yields and the time required for antigen-specific affinity chromatography purification of the immunoglobulins, however, protein A purified antibodies were used in some work. Because DEAE and gentle antibodies demonstrated no significant improvement on nonspecific agglutination, they were not used in further studies.

***Nonspecific agglutination of commercial anti-glucuronidase***

When commercial anti-glucuronidases were located, samples of those antibodies were tried in the latex agglutination assay. It was reasoned that, besides differences in antibody purification, antibody production methods might have an influence on nonspecific agglutination. Using commercial antibodies in the latex agglutination test, it was believed, might make a satisfactory assay and would provide information on the cause(s) of nonspecific agglutinations.

Anti-glucuronidase preparations from Molecular Probes, 5′3′, antigen-specific affinity purification, and protein A purification were compared by using different latexes and by using moderate concentrations for adsorption to the latex. All four antibody preparations yielded nonspecific agglutinations (Table 3). The anti-glucuronidase from Molecular Probes and protein A

purified antibody preparations were more reactive than the anti-glucuronidase from 5'3'. Antigen-specific affinity chromatography purified antibody preparations were slightly more reactive than comparably adsorbed antibody preparations from the commercial sources or from the protein A purified antibody preparations.

All four antibody preparations displayed results that suggested a prozone effect. All the strongly positive reactions centered at approximately 7.8 $\mu$g/ml glucuronidase, with reactions weakening or becoming negative as the enzyme concentration was increased or decreased. Positive results were detected at enzyme concentrations as little as 3.8 ng/ml (not shown in Table 3; the results remained essentially unchanged in the degree of agglutination from the results shown for 0.31 $\mu$g/ml). Positive results at the extreme dilutions were considered non-specific agglutination because latex agglutination tests are theoretically able to detect only about 10 ng/ml under ideal conditions (1). When more immunoglobulin was used to sensitize the latex, stronger reactions and more nonspecific agglutination were noticed.

While it was possible to obtain acceptable results with this batch of sensitized latex using glucuronidase in GBS-BSA buffer as samples, the latex preparation did not provide definitive results when cell lysates were examined (data not shown). The use of cell lysates continued to result in nonspecific reactions. Changes in cell lysis buffers did nothing to alleviate the nonspecific agglutination (discussed in part 3 of this dissertation).

Whereas it was expected that antigen-specific affinity chromatography purified antibody preparations would have displayed more reactivity when less antibody was used to sensitize the latex. But, this was not observed. Antigen-specific affinity chromatography purified antibody preparations should contain more functionally reactive immunoglobulins per unit measure than an equivalent concentration of an antibody preparation purified by using another method.

The reactivities of the commercial anti-glucuronidase preparations were, for the most part,

equal to those detected with the in-house antibody preparations. In addition, all four antibody preparations exhibited similar nonspecific agglutination reactions. Because of time and resource constraints, the commercial sources of anti-glucuronidase were not evaluated for cross-reactivities such as was completed for the antibody preparations made in-house. In discussion with the manufacturers, they revealed that the preparations were protein A purified from rabbits immunized with enzymes from Sigma. While the full characterization of these antibodies would have been informative, the time and costs involved would have been excessive, considering what useful information might have been gleaned. In conclusion, there were no substantial differences in the use of the commercial antibody preparations that warranted their continued use.

***General immunoglobulin induced nonspecific agglutination***

An investigation into the adsorption of nonenzyme antibody preparations on latex was completed because of the continual presence of nonspecific agglutination in the latex assays. If the nonspecific agglutination problems were related to the anti-enzyme preparations, then switching immunoglobulins to unrelated preparations should resolve the problem. Commercial antibodies that had no relation to the project (horse IgG, mouse IgG, and rabbit IgG) were used to determine if the immunoglobulins themselves caused nonspecific agglutination.

The results revealed that immunoglobulins, despite the type or source, can cause nonspecific agglutination (Table 4). Although there were slight differences in the degree of agglutination with the commercial immunoglobulins, all caused a degree of nonspecific agglutination. When protein A purified anti-glucuronidase mixed with normal rabbit serum was used to sensitize the latex, nonspecific agglutination was practically eliminated; reactions with glucuronidase, however, were not present (data not shown). This information reinforced the idea that the immunoglobulin itself, despite the source, contributes directly to nonspecific agglutination. It was concluded that methods for reducing nonspecific agglutination of immunoglobulin sensitized

latex required modification of other parameters, including better blocking of the latex and better assay buffer systems.

## SUMMARY AND CONCLUSIONS

In attempts to eliminate nonspecific agglutination, adsorption of the antibodies to the latex was studied. Kaspar (8) had used staphylococci as the solid-phase indicator system in a coagglutination assay. The use of latex was a new approach to the anti-enzyme assays. The change from a biological solid-phase to the latex solid-phase was suspected of being part of the cause for false-positive reactions. By determining if immunoglobulins were being adsorbed to the latex, and in what amounts, and by evaluating some immunoglobulin-adsorption parameters that resulted in nonspecific agglutination, it was hoped that the causes of nonspecific agglutination could be elucidated.

Different latex brands and different adsorption buffers were investigated. Differences in the adsorption of immunoglobulins to different brands of latex were found. Differences in adsorption buffers and pH, however, were not found. With the information from the immunoglobulin saturation curve, parameters for the adsorption of immunoglobulins became known. From this information, other studies were attempted to try to resolve the problems with nonspecific agglutination.

The effects of antibody purification resulted in no significant differences on the problem of nonspecific agglutination of the latex. The antibodies, despite the method of purification, continued to contribute to nonspecific agglutination. Commercial anti-glucuronidase was tried to further insure that the nonspecific agglutination was not a result of either the production or the methods of purification of the antibodies. Because earlier work with BSA adsorbed on latex displayed no nonspecific reactivity (Part 1), investigation into the effects of irrelevant immunoglobulins on latex was tried in attempts to understand the cause of nonspecific agglutination reactions. These studies revealed that, under the conditions of the assay, even unrelated immunoglobulins resulted in a degree of nonspecific agglutination.

Continual manifestation of nonspecific agglutination is most likely a result of ionic

interactions among individual sensitized latex particles in the suspension. Latex particles are known to have native surface charges that make them interact and agglutinate (2, 13, 14, 15, 25). Nonspecific agglutinations occurred when latex was sensitized with immunoglobulins but not BSA (Table 2). From the other studies presented (Tables 3 and 4), the phenomenon was not limited to immunoglobulins made in-house, but also occurred when other immunoglobulins were used to sensitize the latex. When less immunoglobulin was used, nonspecific agglutination was reduced proportionally, but so was the sensitivity of the assay.

Information on sensitization of latex contends that after a certain adsorption level, additional protein stabilizes latex flocculation (9, 12, 19). The addition of protein normally neutralizes charge interactions and stabilizes the latex suspension (13, 16, 24). This obviously did not occur during the development of the present assays. After sensitizing latex with immunoglobulins, saturating quantities of irrelevant proteins (e.g. BSA) should have imparted stability on the latex suspensions. Because stabilization of the latex was not observed, further explanations for the nonspecific agglutination phenomena were sought, including the study of the assay conditions and the effects of blocking agents, and are presented in the next part.

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TABLE 1. Effects of buffered sodium chloride on unsensitized latex

| Concentration of NaCl in GBS[a] (g/L) | Observation times | | | | | |
|---|---|---|---|---|---|---|
| | 10 min | 30 min | 1 h | 2 h | 3 h | ~18 h |
| 10 | O[b] | ± | 1+ | 2+ | 2+ | 2+ |
| 5 | O | O | O | ± | 1+ | 1+ |
| 2.5 | O | O | O | O | O | ± |
| 1.25 | O | O | O | O | O | O |
| 0.625 | O | O | O | O | O | O |
| 0.313 | O | O | O | O | O | O |
| 0.156 | O | O | O | O | O | O |
| 0.078 | O | O | O | O | O | O |

[a]GBS - glycine buffered saline.
[b]O represents a negative or no change in condition, ± represents a visible but weak reaction, 1+ represents partial flocculation, 2+ represents complete flocculation in that no latex is left in suspension.

TABLE 2. Effects of antibody purification on nonspecific agglutination

| Latex Preparation | Protein coating (mg/ml) | Appropriate enzyme[c] (mg/ml) | | | $dH_2O$ | 1M $KH_2PO_4$ | Commercial enzyme preparations (mg/ml)[d] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.1 | 0.01 | 0.001 | | | 0.1 GUD | 0.1 GAD | 0.1 Tryp |
| Anti-glutamate decarboxylase affinity purified antibody | 0.047[a] | ++[b] | ++ | 0 | 0 | 0 | 0 | ++ | ++ |
| | 0.047 | +++ | ++ | 0 | 0 | 0 | 0 | ++ | ++ |
| | 0.047 | | | | 0 | | + | ++ | +++ |
| Anti-glutamate decarboxylase DEAE purified antibody | 0.141 | +++ | +++ | ++ | ++ | + | ++ | +++ | +++ |
| | 0.094 | +++ | +++ | ++ | + | + | ++ | +++ | +++ |
| | 0.047 | ++ | ++ | 0 | 0 | ± | + | +++ | ++ |
| Anti-glutamate decarboxylase protein A purified | 0.0738 | +++ | +++ | ± | 0 | 0 | ± | +++ | +++ |
| | 0.0369 | +++ | +++ | ± | 0 | 0 | ± | +++ | ++ |
| Anti-glutamate decarboxylase gentle purification | 0.094 | | | | ++ | | +++ | +++ | +++ |
| | 0.047 | | | | + | | ++ | ++ | ++ |

[a]These results are from three separate batches of sensitized latex. The other results are from multiple coatings done during the same batch preparation.
[b]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has a degree of agglutinated particles on magnified examination; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed; ++ reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles; +++ reaction has several agglutinated particles of a moderate size with a relatively clear background; ++++ reaction has one or more agglutinated particle clumps with no macroscopically free particles.
[c]Appropriate refers to using as an antigen the enzyme with the same anti-enzyme sensitized latex.
[d]GUD - glucuronidase; GAD - glutamate decarboxylase; Tryp - tryptophanase.

TABLE 2. Continued

| Latex Preparation | Protein coating (mg/ml) | Appropriate enzyme[a] (mg/ml) | | | $dH_2O$ | 1M $KH_2PO_4$ | Commercial enzyme preparations (mg/ml)[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.1 | 0.01 | 0.001 | | | 0.1 GUD | 0.1 GAD | 0.1 Tryp |
| Anti-tryptophanase affinity purified antibody | 0.047[a] | +++ | ++ | 0 | 0 | 0 | 0 | + | +++ |
| | 0.047 | ++ | + | 0 | 0 | 0 | 0 | ± | ++ |
| | 0.047 | +++ | ++ | 0 | 0 | 0 | 0 | 0 | + |
| Anti-tryptophanase DEAE purified antibody | 0.141 | +++ | +++ | ++ | + | ± | + | ++ | +++ |
| | 0.094 | +++ | +++ | + | ± | ± | + | ++ | +++ |
| | 0.047 | ± | 0 | 0 | 0 | 0 | 0 | 0 | + |
| Anti-tryptophanase protein A purified | 0.0738 | +++ | +++ | 0 | ± | 0 | ± | + | ++ |
| | 0.0369 | +++ | +++ | 0 | 0 | 0 | ± | + | ++ |
| BSA coated blue dyed latex | 0.047 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BSA coated red dyed latex | 0.047 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

TABLE 3. Degree of agglutination in the presence of glucuronidase when three brands of latex were each sensitized with three concentrations of four different anti-glucuronidase preparations

| Latex brand | Antibody source[a] | Protein coating (mg/ml) | Glucuronidase enzyme in GBS-BSA[c] buffer (μg/ml) | | | | | | | | | | | | | | | GBS-BSA buffer |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1000 | 500 | 250 | 125 | 62.5 | 31.3 | 15.6 | 7.81 | 3.91 | 1.95 | 0.98 | 0.49 | 0.24 | 0.12 | 0.06 | 0.03 | |
| Seradyn | Mol. Probe | 0.08 | +[b] | + | + | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | + | + | 0 |
| | | 0.06 | 0 | 0 | 0 | ± | ± | + | + | ± | + | + | ± | + | + | ± | ± | ± | 0 |
| | | 0.04 | 0 | 0 | 0 | 0 | ± | ± | + | ± | ± | ± | ± | + | ± | ± | ± | 0 | 0 |
| | 5′3′ | 0.08 | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | 0 |
| | | 0.06 | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | 0 |
| | | 0.04 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | protein A | 0.08 | ± | ± | ± | + | + | ++ | ++ | ++ | ++ | ++ | ++ | + | + | ± | ± | ± | 0 |
| | | 0.06 | 0 | 0 | 0 | ± | ± | + | + | + | + | ++ | ++ | ± | ± | 0 | 0 | 0 | 0 |
| | | 0.04 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ± | ± | ± | 0 | 0 | 0 | 0 | 0 | 0 |
| | affinity | 0.04 | ± | ± | ± | ± | ± | + | + | + | + | + | + | + | ± | ± | ± | ± | 0 |
| | | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 0.005 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]Mol. Probe - Molecular Probes; 5′3′ - 5Prime→3Prime; protein A - protein A purified antibodies; affinity - antigen-specific affinity chromatography purified antibodies.

[b]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has a degree of agglutinated particles on magnified examination; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed; ++ reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles; +++ reaction has several agglutinated particles of a moderate size with a relatively clear background; ++++ reaction has one or more agglutinated particle clumps with no macroscopically free particles.

[c]GBS-BSA - glycine buffered saline with bovine serum albumin.

TABLE 3. Continued

| Latex brand | Antibody source | Protein coating (mg/ml) | Glucuronidase enzyme in GBS-BSA buffer (μg/ml) | | | | | | | | | | | | | | | GBS-BSA buffer |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1000 | 500 | 250 | 125 | 62.5 | 31.3 | 15.6 | 7.81 | 3.91 | 1.95 | 0.98 | 0.49 | 0.24 | 0.12 | 0.06 | 0.03 | |
| Bangs | Mol. Probe | 0.08 | + | ++ | ++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | ++ | ++ | + | + | ± |
| | | 0.06 | 0 | ± | ± | + | + | ++ | +++ | ++ | ++ | ++ | ++ | ++ | + | + | ± | ± | 0 |
| | | 0.04 | 0 | 0 | 0 | 0 | ± | ± | ± | ± | + | + | + | + | + | ± | ± | ± | 0 |
| | 5′3′ | 0.08 | + | + | + | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + |
| | | 0.06 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 |
| | | 0.04 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | protein A | 0.08 | ± | ± | + | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | + | ± |
| | | 0.06 | 0 | 0 | ± | + | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | ± | 0 |
| | | 0.04 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ± | ± | + | + | + | ± | ± | 0 | 0 | 0 |
| | affinity | 0.04 | 0 | 0 | 0 | ± | + | + | ++ | ++ | ++ | + | + | + | + | ± | ± | ± | 0 |
| | | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 0.005 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

TABLE 4. General immunoglobulin induced nonspecific agglutination

| Immunoglobulins[a] | Protein coating (mg/ml) | Lysis buffer[b] | TPG buffer | GBS-BSA |
|---|---|---|---|---|
| Protein A Purified | 1.0 | 0[c] | 0 | ± |
| Anti-glucuronidase | 0.1 | 0 | 0 | 0 |
| Molecular Probes | 1.0 | +++ | ± | ND[d] |
| Anti-glucuronidase | 0.1 | +++ | 0 | ND |
| Horse IgG | 1.0 | +++ | ++ | + |
| | 0.1 | +++ | | 0 |
| Mouse IgG | 1.0 | + | ++ | ++ |
| | 0.1 | + | | ++ |
| Rabbit IgG | 1.0 | ++ | +++ | ++ |
| | 0.1 | +++ | | +++ |

[a]Horse, mouse, and rabbit IgG were commercial preparations of general immunoglobulins that were not related to detection of *E. coli* enzymes; they may or may not contain any anti-enzyme activity.

[b]Lysis buffer - a phosphate buffered saline system containing EDTA, lysozyme, sucrose, and Triton X-100; TPG - a phosphate saline buffer with gelatin; GBS-BSA - glycine buffered saline with bovine serum albumin.

[c]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has a degree of agglutinated particles on magnified examination; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed; ++ reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles; +++ reaction has several agglutinated particles of a moderate size with a relatively clear background; ++++ reaction has one or more agglutinated particle clumps with no macroscopically free particles.

[d]ND = not done

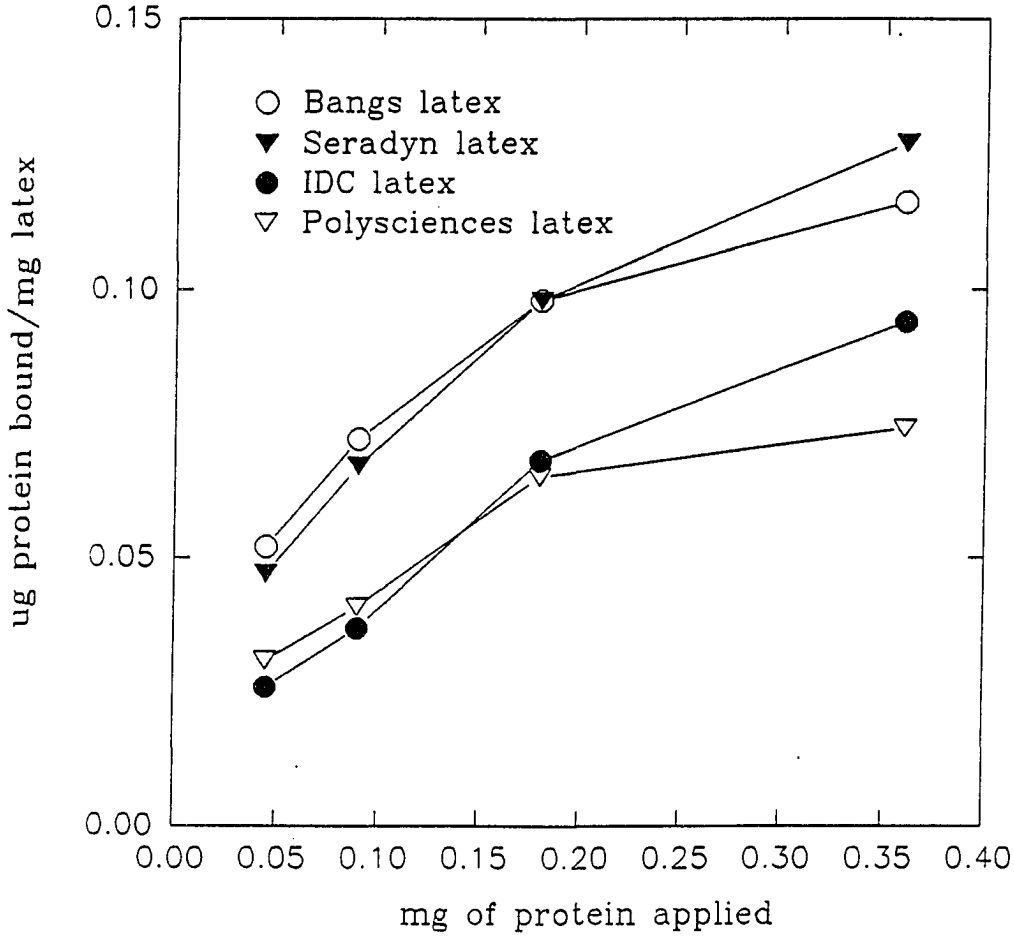


Figure 1. Comparison of protein binding by four brands of latex particles using antigen specific affinity chromatography purified anti-glucuronidase as the protein.

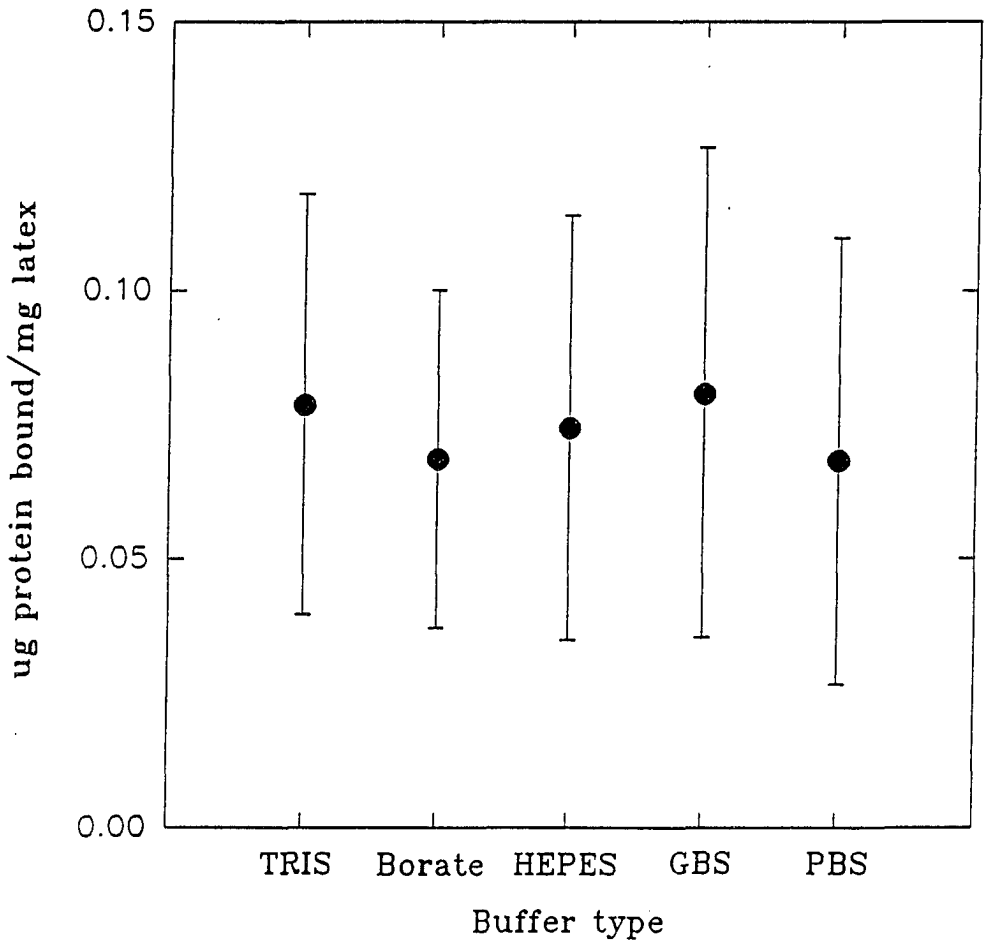


Figure 2. Points represent the average quantities of protein bound for each adsorption buffer used for antibody sensitization of four brands of latex with antigen specific affinity chromatography purified anti-glucuronidase. The experiments were represent the averages of twelve determinations per buffer. Maximum and minimum values are shown, as well as the average and ± 2 standard deviations (95% limits).

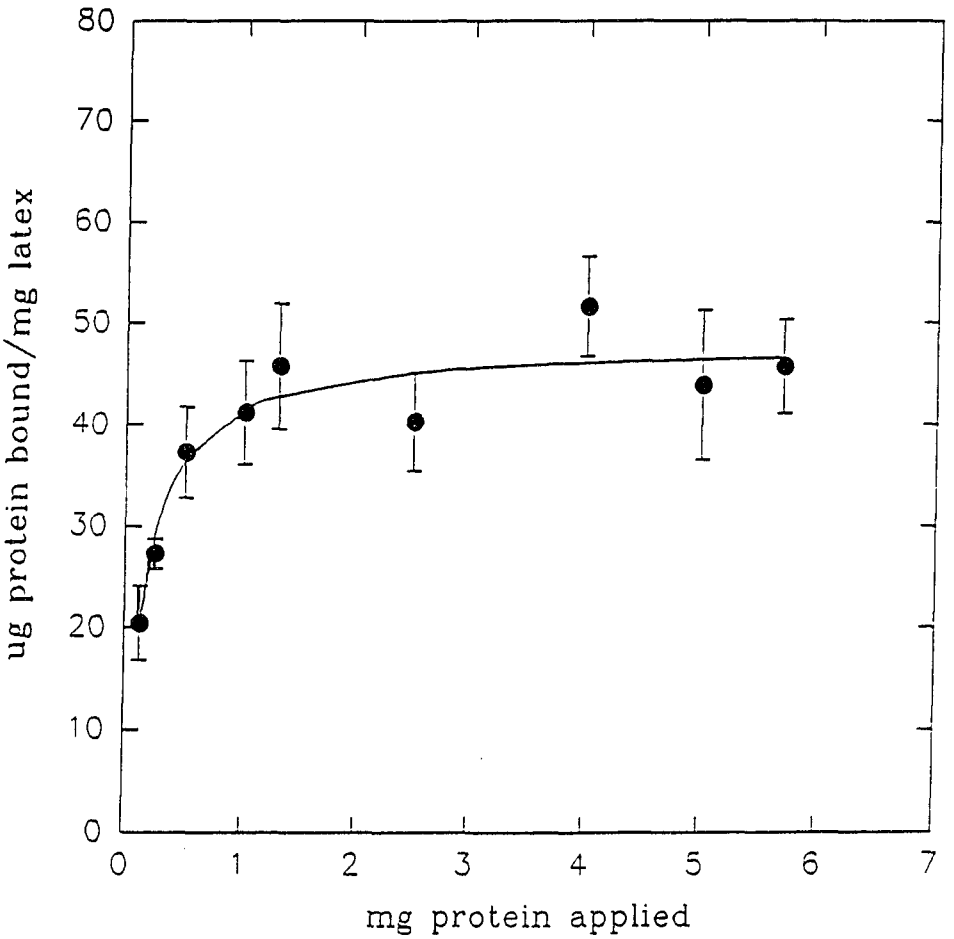


Figure 3. Relationship between quantity of protein used and quantity bound to latex. Data shown are the average values of a minimum of three determinations per point using protein A purified anti-glucuronidase as the protein.

## PART 4. EFFECTS OF BUFFERS AND BLOCKING AGENTS ON SENSITIZED LATEX

## INTRODUCTION

Results from the initial work on the development of the latex agglutination tests revealed serious nonspecific agglutination reactions that limited the specificity of the assays. Investigation into adsorption of immunoglobulins revealed that, the latex particles adsorbed immunoglobulins under a variety of conditions. The adsorbed immunoglobulins, however, significantly contributed to nonspecific agglutination under the conditions of assay performance. The causes of nonspecific agglutination, resulting from immunoglobulin adsorption to latex particles, could be a result of either the ionic conditions of the assay system or incomplete blocking of the latex particles with irrelevant proteins. One recommended procedure for optimizaton of a latex assay (47) is adjustment of the assay buffer pH and ionic strength.

Another area for examination concerns the blocking of reactive sites on the latex particles. A possible explanation for the cause(s) of nonspecific agglutination is that blocking is incomplete and leaves open, unreacted sites on the latex available for charge-induced reactions. Two causes for incomplete blocking of the remaining reactive sites could be proposed: either steric hindrance by complete saturation with immunoglobulins failed to allow the blocking agents to adsorb on the latex surface or blocking agents, such as BSA, failed to completely cover open areas on the latex surface. If the original hypothesis was correct, then less immunoglobulins on the latex should allow saturation of the latex with blocking protein without significant steric hinderance from the immunoglobulins. Results previously presented (Part 2, Table 4), however, showed that this is not true. The latter hypothesis, that globular proteins failed to completely cover open areas, was suggested from reports from work on other solid-phase assay systems (10, 19, 45, 46).

In attempts to optimize the latex agglutination assay and eliminate nonspecific reactions, many different procedures were tried. These are described in this introduction, but no data are presented because the results showed that all these methods were ineffective. They are mentioned here, however, to provide a basis for the experiments that are described in more detail in this part of

the dissertation. Washing latex in alcohol before adsorption of the antibodies was accomplished as suggested by Kakabakos et al. (27, 28) without any obvious difference in protein binding or agglutination assay results. Tween 20 (37, 47, 56), normal rabbit serum, and polyvinylpyrrolidone (24; molecular weight 40,000; Sigma, product number PVP-40) were tried as blocking agents for sites without adsorbed antibody. Results from using these agents in the assays did not warrant further work. Another detergent, GAFAC® RE-610 (Rhône-Poulenc, Inc., Princeton, NJ) had been successfully used previously (13, 36), but it had no significant effects on assay performance in my tests. Protein A-coated latex (46) was used unsuccessfully to capture the anti-enzyme antibodies. Adsorption and covalent coupling of the antibody preparations to carboxyiate modified latex particles (3, 12, 25, 47, 57) were also tried without the sought after reduction of nonspecific agglutination reactions.

To continue investigations of the causes of nonspecific agglutination, the conditions for the performance of the latex tests were examined. In addition to those described in the previous paragraph, the effects of buffers and blocking agents to reduce or eliminate nonspecific agglutination were studied. The establishment of functional parameters for the latex agglutination assays were determined.

# MATERIALS AND METHODS

## Common Procedures Used in this Project

### *Stock Cultures and Media*

The bacterial cultures were obtained from either Dr. Paul A. Hartman or from the Department of Microbiology, Immunology, and Preventive Medicine stock culture collection, Iowa State University, Ames, IA. The cultures were grown on tryptlcase soy agar and identities were confirmed by using API-20E kits (bioMérieux, Hazelwood, MO, product number 886-010258).

All media were products of Difco Laboratories (Detroit, MI), unless otherwise noted. Enzyme induction broth (EIB) was made according to the formulation of Kaspar (29), as follows: 3.56 g lauryl tryptose broth (Difco); 0.015 g 4-methylumbelliferyl-ß-D-glucuronide (MUG; Hach Company, Ames, IA, product number 21844-21); 0.05 g L-glutamic acid (Oxoid Limited, Basingstoke, Hampshire, England, product code L124); 100 ml distilled water; pH 6.8-7.0.

### *Cell lysis*

To obtain immunologically reactive enzymes from the cultures, the method of Kaspar et al. (30) was used. First, cells were sedimented from 0.5 ml of a 24-h culture grown in EIB by centrifugation in a microcentrifuge (Fisons, England, model Microcentaur) for 5 min at approximately 11,300 × *g*. The supernate was removed and the cell pellet was suspended to the original volume in lysis buffer (25 ml of 0.2 M $Na_2HPO_4$, 25 ml of 0.2 M $NaH_2PO_4$, 8 g of sucrose, 0.93 g of EDTA, 50 µl of Triton X-100, total volume to 50 ml with $dH_2O$, pH 7.0). RNAse solution (0.5 ml; 20 mg/ml bovine pancreas ribonuclease A, 0.5 µl/ml Tween 20, and 30 mg/ml BSA in 0.01 M phosphate buffered saline [0.01 M $NaH_2PO_4$-$H_2O$, 0.155 M NaCl], pH 7.0) and 15 µl of lysozyme solution (10 mg/ml lysozyme in phosphate buffered saline [0.01 M $NaH_2PO_4$-$H_2O$, 0.155 M NaCl], pH 7.0) were added. The pellet was suspended by mixing on a vortex mixer and incubated for 20 to 30 min at 37°C. Cellular debris was pelleted by centrifugation and the supernatant was used for the test material.

## Sensitization of Latex to Determine the Effects of Assay Conditions on Nonspecific Agglutination

***Sensitization of anti-glucuronidase latex for testing the effects of different assay buffers on nonspecific agglutination***

In three separate experiments, 0.5 ml of 1% sensitized latex was prepared. A sample of 0.8-$\mu$m diameter yellow dyed polystyrene latex (Bangs Laboratories, Indianapolis IN) was cleaned by centrifugation washing. The latex was sensitized by using antibody concentrations of 6, 3, 2, 1, and 0.5 mg/ml in Tris/azide buffer (0.03 M Tris, 0.05% sodium azide, pH 7.4). One milliliter of protein A purified anti-glucuronidase antibody preparation was added to an appropriately labeled 2.0-ml centrifuge tube. Ten milligrams of washed latex were then added, the volume increased to 1.5 ml total volume with buffer, and the tubes mixed by inversion several times. The latex/antibody suspensions were incubated at 37°C overnight on a platform rocker (Thermolyne Speci-Mix, model M26125), pelleted by centrifugation, washed once with Tris/azide buffer and suspended to a volume of 0.5 ml with Tris/azide buffer. Latex-bound proteins were determined on a sample of the latex suspension (see Latex Bound Protein Determinations). One milliliter of 1 mg/ml bovine serum albumin in buffer was added to block unreacted sites on the latex and the latex suspensions were incubated at room temperature (approximately 27°C) overnight on a platform rocker. After the second incubation, the latex suspensions were again pelleted by centrifugation, washed twice in buffer, and suspended to a volume of 0.5 ml (1% [w/v] latex) with buffer containing 1 mg/ml BSA and 0.1% thimerosal. The sensitized latex was stored at 4°C.

Buffers of several compositions and ionic strengths were then used as assay buffers. These buffers included TPG buffer (7.8 g/L $NaH_2PO_4 \cdot 2H_2O$, 1 g/L NaCl, 2 g/L gelatin, pH 6.6), glycine buffered saline (7.3 g/L glycine, 10 g/L NaCl, pH 8.2), Medcalf's buffer (21 g/L borate, 22.37 g/L KCl, 1% BSA, 33.8 g/L polyethylene glycol, pH 8.0) and Tris/azide-BSA (0.03 M Tris, pH 7.4, with 0.1% BSA and 0.05% sodium azide). The ionic strength of each buffer was adjusted by the addition

of NaCl if necessary, according to the formula:

$$\frac{1}{2}\sum cZ^2$$

in which c = concentration and Z = valence. TPG buffer with ionic strengths of 0.4, 0.5, 0.6, 0.7, 0.8, 1.0, 1.25, 1.5, 1.75, and 2.0, Medcalf's buffer with ionic strengths of 0.175, 0.35, 0.7, and 1.4, GBS with ionic strengths of 0.57 and 0.7 and Tris/azide buffer with an ionic strength of 0.045 were used to wash and suspend a sample of latex. Portions of the sensitized latex preparations were washed in the assay buffer before being resuspended to 1% (w/v). The latex was then used to conduct agglutination assays with some of the following samples: cell lysates from *E. coli* B, *E. coli* K12, *E. coli* 222, *E. coli* 329, *Enterobacter agglomerans*, glucuronidase enzymes at 0.1 and 0.01 mg/ml, lysis buffer, and lysis buffer without lysozyme.

***Sensitization of anti-glucuronidase latex for testing the effects of saline on nonspecific agglutination***

Antibody preparations containing 0.04, 0.05, 0.06, and 0.07 mg/ml were prepared from four different anti-glucuronidases: antigen-specific affinity chromatography purified, protein A purified, Molecular Probes (Molecular Probes, Inc., Eugene, OR, product number A-5790), and 5'3' (5Prime$^-$ 3Prime Inc., Boulder, CO, product number 5307-246468). Protein estimates were made by using $A_{280}$ values and an extinction coefficient of 1.4 (8, 22). Ten milligrams of 0.81-$\mu$m diameter washed yellow dyed polystyrene latex (Bangs Laboratories) were added to 1.75 ml of each antibody preparation (total volume 2 ml) and mixed by inversion. The latex suspensions were incubated for 1 h at 37°C with intermittent mixing by inversion followed by 1 h stationary. Tris/azide buffer (0.25 ml) with 4 mg/ml BSA was added and allowed to incubate with the latex suspension stationary, overnight at 4°C. The next day, the latex was sedimented by centrifugation until a latex pellet formed and the supernate was clear, using a Sorval model RT6000B centrifuge at 3400 RPM (2410 × *g*) and 10°C (Sorval Instruments, DuPont Co., Newton CT). All but 0.5 ml of the supernate was removed and the latex pellet was resuspended in the remaining supernate. Portions of the

sensitized latex suspensions (200 $\mu$l) were transferred to 2.0-ml microcentrifuge tubes and the volumes increased to approximately 1 ml with filtered buffer solution. Buffers used consisted of Tris/azide, borate (0.34 M boric acid, pH 8.0, with 0.1% BSA and 0.05% sodium azide), glycine buffered saline (7.3 g/L glycine, 10 g/L NaCl, pH8.2), and phosphate buffered saline (0.01 M $NaH_2PO_4 \bullet H_2O$, 0.155 M NaCl, pH 6.6). The sensitized latex suspensions were washed three times by centrifugation washing in the appropriate buffer before use. The samples for testing consisted of 0.43, 0.26, 0.17, 0.09, 0.04, and 0.02 M NaCl solutions without the presence of antigens (see "Performance of Agglutination Assays" previously described).

***Sensitization of anti-glutamate decarboxylase latex for testing the effects of saline on nonspecific agglutination***

Protein A purified anti-glutamate decarboxylase antibodies at concentrations of 0.1, 0.08, 0.06, and 0.04 mg/ml were prepared in 12 × 75 mm test tubes in Tris/azide buffer (0.03 M Tris, pH 7.4, 0.1% BSA, 0.05% sodium azide). Ten milligrams of washed 0.8-$\mu$m diameter blue dyed polystyrene (Bangs Laboratories) was added to 1.75 ml of each antibody preparation (total volume 2 ml) and mixed by inversion. The latex suspensions were incubated for 1 h at 37°C with intermittent mixing by inversion followed by 1 h stationary. Tris/azide buffer (0.25 ml) with 4 mg/ml BSA was added and allowed to incubate, with the latex suspension stationary, overnight at 4°C. The latex suspension was washed three times before the latex was suspended in assay buffer at 1% (w/v). Four different types of assay buffers were used: glycine buffer (0.03 M, pH 8.2, with 0.1% BSA and 0.05% sodium azide), phosphate buffer saline (0.1 M $NaH_2PO_4$-$H_2O$, 0.155 M NaCl, pH 6.6, with 0.1% BSA and 0.05% sodium azide), borate buffer (0.34 M boric acid, pH 8.0, with 0.1% BSA and 0.05% sodium azide), and Tris buffer (0.03 M, pH 7.4, with 0.1% BSA). The sensitized latex suspensions were washed three times by centrifugation washing in the appropriate buffer before use. The samples for testing consisted of 0.43, 0.26, 0.17, 0.09, 0.04, and 0.02 M NaCl solutions without the presence of antigens (see "Performance of Agglutination Assays" previously described).

**Preparation of Anti-glutamate Decarboxylase Latex with Multiple Blocking Agents**

Antigen-specific affinity chromatography purified anti-glutamate decarboxylase was adjusted to 0.5 and 2.25 mg/ml protein concentration using $A_{280}$ values and an extinction coefficient of 1.4 (8, 22). In addition, two preparations of antigen-specific affinity chromatography purified anti-glutamate decarboxylase was prepared containing ß-casein (Sigma product number C-6905; 11, 26). The first preparation consisted of 0.5 mg/ml antibody and 0.02 mg/ml ß-casein. The second preparation consisted of 2.25 mg/ml antibody and 0.075 mg/ml ß-casein. Two milliliters of anti-glutamate decarboxylase were added to six 12 × 75 mm test tubes. Two milliliters of anti-glutamate decarboxylase-casein were added to two 12 × 75 mm test tubes. The tubes were prewarmed to 37°C and ten milligrams of either 0.8 $\mu$m or 0.2 $\mu$m diameter dialysis cleaned blue dyed latex (Bangs Laboratories) were added to each tube. The tubes were covered with Parafilm (American National Can, Greenwich, CT), mixed by inversion several times, and incubated at 37°C for 2.5 h; they were mixed by inversion approximately every 15 min for the first hour.

The six anti-glutamate decarboxylase sensitized latex preparations were sedimented by centrifugation, until a latex pellet formed and the supernate was cleared, using a Sorval model RT6000B centrifuge at 3400 RPM (2410 × *g*) and 10°C (Sorval Instruments, DuPont Co., Newton CT). The times of centrifugation varied from a few minutes, when using 0.8-$\mu$m diameter latex, to over three h, when using 0.2-$\mu$m diameter latex. Tris/azide buffer (1.25 mls) containing either ß-casein (0.5% w/v) and thimerosal ([sodium ethylmercurithiosalicylate] 0.2% w/v; 31), ß-casein (0.33 mg) and BSA (0.165 mg), or ß-casein (5 mg/ml) and glycerol (5%; Fisher Scientific, product number G33-500) were used as blocking agents for the three anti-glutamate decarboxylase sensitized latex preparations. The anti-glutamate decarboxylase-casein sensitized latex preparation had no additional blocking agents added. The suspensions were incubated overnight at approximately 4°C.

All the sensitized latex preparations were sedimented by centrifugation, until a latex pellet

formed and the supernate was cleared, using a Sorval model RT6000B centrifuge at 3400 RPM (2410 × *g*) and 10°C (Sorvai Instruments). The latexes were then washed twice in buffer (0.03 M Tris, 5 mg/ml BSA, 5% glycerol, 0.016 mg/ml ß-casein, 0.05% sodium azide, pH 8.2) before suspending to 1% (w/v) in the wash buffer. The latex suspensions were then tested against several sources of antigens and controls using the slide agglutination procedure discussed previously.

**Preparation of Sensitized Latex to Determine the Effects of Sodium Salicylate on Nonspecific Agglutination**

Antigen-specific affinity chromatography purified decarboxylase and anti-tryptophanase were adjusted to approximately 6 mg/ml protein concentration using $A_{280}$ values and an extinction coefficient of 1.4 (8, 22). Two milliliters of 6, 3, 1.5 and 0.5 mg/ml antibody preparation were added to 12 × 75 mm test tubes. The tubes were prewarmed to 37°C and twenty milligrams of 0.2 $\mu$m diameter dialysis cleaned dyed latex (Bangs Laboratories) were added to each tube. The test tubes were covered with Parafilm (American National Can, Greenwich, CT) and mixed by inversion several times. The latex suspensions were incubated at 37°C for 2 h; they were mixed by inversion approximately every 15 min for the first hour.

A sample of each latex suspension (0.055 ml) was removed to determine latex-bound protein as previously described. The sensitized latex preparations were sedimented by centrifugation, until a latex pellet formed and the supernate was cleared, using a Sorvai model RT6000B centrifuge at 3400 RPM (2410 × *g*) and 10°C (Sorvai Instruments, DuPont Co., Newton CT). Tris/azide buffer (0.5 ml) containing 10 mg/ml ß-casein and glycerol (5%; Fisher Scientific, product number G33-500) were used as blocking agents. The suspensions were incubated overnight at approximately 4°C.

Sensitized latex preparations were sedimented by centrifugation, until a latex pellet formed and the supernate was cleared, using a Sorval model RT6000B centrifuge at 3400 RPM (2410 × *g*) and 10°C (Sorval Instruments). The latexes were then washed twice in buffer (0.03 M Tris, 5 mg/ml

BSA, 5% glycerol, 0.016 mg/ml casein, 0.05% sodium azide, pH 8.2) before suspending to 1% (w/v) in fresh buffer. The latex suspensions were then tested against several cell lysates using the slide agglutination procedure discussed previously.

## RESULTS AND DISCUSSION

### Effects of Assay Conditions on Nonspecific Agglutination

***Nonspecific agglutination of anti-glucuronidase latex in different assay buffers***

Results from the sensitization of latex (Part 2) suggested that immunoglobulin sensitized latex undergoes nonspecific agglutination reactions. The cause of nonspecific agglutination could be a result of the conditions under which the sensitized latex is tested. To investigate the effects of the assay conditions on nonspecific agglutination of sensitized latex, different assay buffers, with different saline concentrations, were tried.

Table 1 presents the effects of ionic strength on the agglutination reaction. When latex, sensitized with antibody preparations that covered the entire protein saturation curve (Part 1), was suspended in buffers of different ionic strengths, the agglutination reaction changed, depending on the electrolyte conditions. In general, the higher the ionic strength of the buffer, the greater the effect on the latex that had been sensitized with large amounts of antibody (3 and 6 mg/ml). The latex preparations that had the larger amounts of antibody tended to become less reactive and not display nonspecific agglutination. This is in direct contrast to the flocculation detected with unsensitized latex (Part 2).

Sensitized latex in glycine buffered saline (a commonly used buffer for latex agglutination assays; 4, 5, 6, 9, 11, 34, 48, 51), exhibited nonspecific agglutination with all the samples without any significant differences resulting from the variation in ionic strengths. TPG is a phosphate buffer with gelatin described by Bangs (company monograph) for use in latex agglutination tests. When TPG was used as the assay buffer, the ionic strength influenced the nonspecific agglutination of the reaction more than with the other two buffers in this set of experiments. As the ionic strength of the buffer was increased, both nonspecific and specific agglutinations were neutralized on the latex sensitized with the greater immunoglobulin concentrations. Medcalf et al. (13, 36, 52) described a borate buffer that was used successfully in several studies. This buffer yielded results similar to

those obtained with glycine buffered saline. The overall conclusion derived from the results presented in Table 1, however, was that with these three buffers, changes in the ionic strength did little to eliminate nonspecific agglutination reactions.

Singer and Plotz (49) reported that sodium chloride, near normality (0.31 to 1.25%), was necessary for optimal agglutination reactions. Kondo et al. (33), Price et al. (43), Medcalf et al. (36), and Deželić et al. (15) all stated that the ionic strength of the reaction buffer effected agglutination reactions. Whereas all the buffers had at least normal saline concentrations, buffers with different ionic strengths (on the basis of saline concentration) were used to discern if nonspecific agglutination could be eliminated. It was reasoned that if the nonspecific agglutination was related to the ionic influences of the buffers, changes in the buffer ionic strengths might affect nonspecific agglutination. In these tests, buffers with greater ionic strengths did not sufficiently eliminate nonspecific agglutination but did affect the overall strength of the agglutination reactions in the assays.

***Nonspecific agglutination of anti-glucuronidase latex in saline***

Because the effect of different buffers and saline concentrations did not eliminate nonspecific agglutination reactions, the effects of saline on sensitized latex without antigens were examined.

There was essentially no difference in nonspecific agglutination on the basis of antibody purification or source (Table 2). Within each buffer, all four sources of antibody preparations reacted similarly with respect to nonspecific agglutination reactions. Glycine and borate buffers exhibited the least amount of nonspecific agglutination reactions. Tris/azide buffer yielded nonspecific agglutination reactions consistent with increasing sodium chloride and sensitizing protein concentrations. Phosphate buffer, with varying concentrations of sodium chloride, induced nonspecific agglutination in all the latex preparations.

In general, nonspecific agglutination reactions were more evident as the pH of the buffer solutions decreased (Table 2). This is consistent with the recommendation (47) that the pH during

latex agglutination should not be at or near the isoelectric point of the antibody (rabbit IgG has an isoelectric pH of approximately 7.8; 40). In the studies presented here, however, those buffers with a pH below 7.8 were the most prone to induction of nonspecific agglutination reactions.

Results of tests with the latex suspended in the glycine buffer against cell lysates from three organisms (*E. coli* B, *E. coli* 222, and *Enterobacter* sp.), were not acceptable (data not shown). In general, the reactivity with cell lysates was weak and not specific. The weak results might be attributed to the low concentration of immunoglobulins adsorbed to the latex particles.

***Nonspecific agglutination of anti-glutamate decarboxylase latex in saline***

These experiments were conducted to confirm that the results just described on the effects of saline on latex sensitized with anti-glucuronidase were similar when a different antibody preparation was used. In this analysis, the effects of buffer pH and immunoglobulin concentration used for adsorption also were evaluated.

Nonspecific agglutination reactions in these assays continued to be related to the pH of the assay buffer (Table 3). When glycine buffer was used, nonspecific agglutination increased when either ionic strength or immunoglobulin concentration on the latex particles were increased. The results of nonspecific agglutination using borate buffer were similar to the results obtained with anti-glucuronidase preparations; nonspecific agglutination reactions were reduced in both severity and frequency. The Tris buffer at pH at 6.0, however, was generally less prone to nonspecific agglutination reactions than the Tris/azide buffer at pH 7.2 used in the previous anti-glucuronidase preparations. Phosphate buffer at pH 6.6 induced strong nonspecific agglutination reactions similar to those obtained in the previous anti-glucuronidase experiments. When the pH of the phosphate buffer was raised to 8.1, however, nonspecific agglutination reactions were eliminated (Table 3).

Whereas the pH of the assay conditions appeared to affect nonspecific agglutination, simple procedures, such as buffer composition or salt content, did not appreciably eliminate nonspecific agglutinations. Applying a change of buffer pH to the actual cell lysis conditions for reduction of

nonspecific agglutination was difficult. The cell lysis procedure developed by Kaspar et al. (30) imparted another set of parameters affecting nonspecific agglutination reactions for which neither pH nor buffer composition alone could compensate (see Lysis Buffer discussed later in this part).

The results of the effects of different buffer compositions and pHs applied to cell lysates also was consistent with the results from the previous anti-glucuronidase latex results (data not shown). Cell lysates continued to have nonspecific agglutination reactions in the glycine assay buffer being used. Insufficient latex remained to test the effects of the Tris or phosphate (pH 8.1) assay buffers with the cell lysates, but it would be expected that the nonspecific reactions also would be present in those assays. It had become evident that the assay buffers alone would not eliminate the nonspecific agglutination reactions and that other areas were more in need of further research.

**Anti-glutamate decarboxylase sensitized latex with multiple blocking agents**

Results from sensitization of latex with immunoglobulins and from testing the sensitized latex revealed that sensitized latex was prone to nonspecific agglutination and that changes in the assay conditions alone would not eliminate the nonspecific agglutination. Introductory work on the development of these latex agglutination assays included some investigation of the effects of blocking agents on nonspecific agglutination. Although the early work did not reveal any useful findings on the effects of the blocking agents, the choice of blocking agents kept recurring as a potential method to eliminate nonspecific agglutination. In addition, the effects of using smaller diameter particles that had less total surface area also were examined.

The effects of using different blocking agents on 0.8- and 0.22-$\mu$m diameter latex particles were evaluated over the course of several experiments. Casein coadsorbed with the antibody, a casein-thimerosal buffer, a casein-BSA buffer, and casein and glycerol as blocking agents, with a range of antibody concentrations, were all tried as methods to overcome nonspecific agglutination. Preliminary results with both commercial enzymes (data not shown) and cell lysates (Table 4) suggested that nonspecific agglutination could be significantly reduced with casein as a blocking

agent. The combination of ß-casein and glycerol or BSA, used with the smaller 0.2-$\mu$m diameter latex particles, appeared to give good results with minimal nonspecific agglutination.

Kenna et al. (31) reported that casein with thimerosal was a better agent for blocking nonspecific reactions than Tween 20. In addition, Vogt et al. (55) tested nine different proteins for their abilities to block nonspecific binding in a microtitration-plate EIA assay and concluded that casein and dry skim milk were the most effective proteins. Although Kenna et al. (31) and Vogt et al. (55) reported that casein was a better blocking agent than BSA, others (44) have reached the opposite conclusion. Hechemy et al. (23) also observed that nonspecific agglutination, in their latex agglutination assay for *E. coli* antigens, was eliminated by using fatty acid-free BSA. In addition, detergents, such as Tween 20 and Triton X-100, are often used to reduce nonspecific agglutination (18, 31, 37, 47, 56). Neither the use Tween 20 during latex preparation (data not shown) nor the presence of Triton X-100 or Tween 20 in the lysis buffers, however, appeared to reduce nonspecific agglutination in the present study.

The use of the smaller 0.2-$\mu$m diameter latex particles and the ß-casein and glycerol blocking agents were used to develop the other anti-enzyme agglutination assays. Overall, the other anti-enzyme latex agglutination tests yielded similar results. Although sporadic nonspecific agglutination still occurred, the assays were generally able to distinguish the appropriate reactions from cell lysates of control organisms. Further investigations using these approaches were made.

**Determination of the Effects of Sodium Salicylate on Nonspecific Agglutination**

Because of the continued sporadic presence of nonspecific agglutination with the 0.2-$\mu$m diameter latex particles and the ß-casein and glycerol blocking agents, sodium salicylate was evaluated for use in the assay. It was recommended in a discussion with Dr. Bruce Shull (Seradyn, Indianapolis IN; personal communication) that sodium salicylate be tried, although none of the literature reviewed had mentioned the used of sodium salicylate in latex agglutination tests. The action of sodium salicylate on nonspecific agglutination is not known, but it has been used as a

protein blocking agent in other immunoassays (1, 21, 42). Although chaotropic agents (14, 47, 50) had been investigated earlier (data not shown), their use had not been encouraging. It had been found that low concentrations of agents did not reduce nonspecific agglutination reactions and that greater concentrations of the agents reduced or eliminated the desired agglutination reaction. Sodium salicylate had not been included in the early work on chaotropic agents. Because of the difference in mode of action, it was tested to determine if it would reduce nonspecific agglutination reactions.

The effects of sodium salicylate on nonspecific agglutination reactions were varied (Table 5). Anti-tryptophanase latex responded well to the presence of sodium salicylate at 100 mM and nonspecific agglutinations were significantly reduced. The specific agglutination reactions, however, also were retarded, but a distinction between positive verses negative reactions was possible. With anti-glutamate decarboxylase latex, however, the effects of 100 mM sodium salicylate were not as pronounced; the anti-glutamate decarboxylase latex showed a general decrease in both specific and nonspecific agglutination reactions. Although a lysis buffer control was not included, the nonspecific agglutination reactions of lysis buffer without cellular material would not be expected to exceed the results observed with the cell lysate from the *Proteus* sp.; there was only minimal growth of *Proteus* in the enzyme induction broth.

The results demonstrated that sodium salicylate could be added to the latex agglutination reactions to control nonspecific agglutination reactions without eliminating the specific agglutination reactions. When combined with the smaller latex and the casein-glycerol blocking agents, it appeared that a suitable latex agglutination assay was near at hand.

**Lysis buffers**

Because some latexes were produced that appeared to work when commercial enzymes in buffers were used, but had nonspecific agglutination reactions when cell lysates were used, the lysis buffer was considered a part of the problem. Several other lysis procedures were tried to determine

if they would reduce nonspecific agglutination reactions with the sensitized latex reagents. Takei et al. (51) also detected nonspecific agglutination in the absence of antigen when they used chromatographically purified IgG fractions of polyclonal rabbit antibodies. To eliminate nonspecific agglutination, they studied fifteen antigen extraction reagents. Although none of the lysis procedures of Takei et al. (51) were tested (this work preceeded publication of their research), a number of other lysis procedures were examined with the goal of eliminating nonspecific agglutination.

To study the effects of lysis buffers on nonspecific agglutination reactions, the buffering agents in the lysis buffer were changed from those used by Kaspar (29). Lysis systems with the buffering agents changed to HEPES, glycine, or borate were tried; other components of the lysis buffer (lysozyme, EDTA, etc.) were not changed. None of the different buffering agents significantly affected nonspecific agglutination reactions (data not shown).

Other cell lysis procedures were also tested. A polymyxin B cell lysis buffer, previously used in a latex agglutination assay (20), was evaluated. In addition, a cell lysis procedure that uses dithiothreitol (35), and a second lysozyme method using Tris/EDTA/NaCl buffer and sodium dodecyl sulfate (2), also were examined. No significant improvement in reduction of nonspecific agglutination reactions was noted with any of the cell lysis procedures (data not shown).

To obtain maximum immunologically reactive enzymes from bacterial cells, Kaspar (29) also tried several different extraction protocols and concluded that his lysozyme method was the most suitable. It has been established, however, that lysozyme will bind immunoglobulins and cause cross-linking (17, 39). Although lysozyme in the lysis buffer did have some affect on the general strength of the agglutination reaction (Table 1), lysozyme was not itself responsible for nonspecific agglutination reactions in this study.

## SUMMARY AND CONCLUSIONS

Attempts to eliminate nonspecific agglutination through adjustment of the assay conditions were only partially successful. Although the effects of saline and pH on the latex assays provided some very useful information, they were not able to reduce nonspecific agglutination reactions to a level that made the assay sensitive and specific. Acceptable reduction of the nonspecific agglutination reactions was achieved with 0.2-$\mu$m diameter latex particles and the ß-casein and glycerol blocking agents. Whereas casein has been used as an effective blocking agent in other immunoassays, this is the first known use of casein to block a latex agglutination assay. (Dr. Bruce Shull [Seradyn, Indianapolis IN; personal communication] did not recommend use of casein for blocking). The effectiveness of ß-casein and glycerol as blocking agents support the hypothesis that BSA was not effectively blocking reactive sites on the latex, leaving exposed latex open for reactions with the immunoglobulins.

Another way that reactive sites might become available on the latex would be from the desorption of proteins from the latex surface. Desorption of passively bound protein has been reported a cause of problems in EIA (41, 54) and latex agglutination assays (7). Up to 68% of protein adsorbed to latex can later desorb (53), leaving reactive spaces on the solid-phase that can contribute to nonspecific agglutination. To try to compensate for the effects of desorption, inert protein (e.g. BSA) was used in both the assay and the lysis buffer. Any protein that desorbed during the performance of the assay would be readily replaced with protein that was present in excess in the assay and lysis solutions.

Although some nonspecific agglutination reactions were still occuring, the latex assays were thought suitable for more extensive evaluation. A more complete evaluation of the sensitivities and specificities of the latex reagents was deemed necessary. From the extensive evaluation of the latex reagents, further work might be planned.

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TABLE 1. Agglutination reactions with cell lysates, glucuronidase, and lysis buffers when three assay buffers of various ionic strengths were used with latex coated at six concentrations with protein A purified anti-glucuronidase

| Buffer | Ionic strength | Protein coating (mg/ml) | Cell lysates[b] | | | | Glucuronidase (mg/ml) | | Lysis buffer | Lysis buffer w/o lysozyme |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *E. coli* B | *E. coli* K12 | *E. coli* #222 and #329 | *Enterobacter* sp. | 0.1 | 0.01 | | |
| Glycine buffered saline | 0.57 | 6 | +[a] | + | | | ++ | +++ | + | |
| | | 3 | ++ | + | | | ++ | +++ | + | |
| | | 1.5 | + | + | | | ++ | +++ | + | |
| | | 0.75 | ++ | + | | | + | +++ | + | |
| | | 0.38 | +++ | +++ | | | ++ | +++ | ++ | |
| | | 0.18 | +++ | +++ | | | ++ | +++ | ++ | |
| | 0.7 | 6 | + | | ± | ± | +++ | +++ | ± | ± |
| | | 3 | ++ | | + | + | ++ | +++ | ++ | ++ |
| | | 1.5 | +++ | | +++ | + | +++ | ++ | +++ | ++ |
| | | 0.75 | +++ | | +++ | +++ | +++ | +++ | +++ | + |
| | | 0.38 | +++ | | +++ | +++ | +++ | +++ | +++ | +++ |
| | | 0.18 | +++ | | +++ | ++ | +++ | ++ | +++ | ++ |
| | 0.8 | 6 | ++ | | + | + | +++ | +++ | + | |
| | | 3 | +++ | | + | + | ++ | +++ | + | |
| | | 1.5 | +++ | | +++ | + | +++ | +++ | +++ | |
| | | 0.75 | + | | +++ | + | +++ | +++ | + | |
| | | 0.38 | +++ | | ++ | ++ | +++ | +++ | ++ | |
| | | 0.18 | + | | + | + | +++ | +++ | + | |

[a]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has some agglutinated particles on magnified examination; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed; ++ reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles; +++ reaction has several agglutinated particles of a moderate size with a relatively clear background; ++++ reaction has one or more agglutinated particle clumps with no macroscopically free particles.
[b]Numbers and letters refer to strain used (for reference only).

TABLE 1. (continued)

| Buffer | Ionic strength | Protein coating (mg/ml) | Cell lysates | | | | Glucuronidase (mg/ml) | | Lysis buffer | Lysis buffer w/o lysozyme |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *E. coli* B | *E. coli* K12 | *E. coli* #222 and #329 | *Enterobacter* sp. | 0.1 | 0.01 | | |
| TPG | 0.4 | 6 | ++[b] | + | | | +++ | +++ | + | |
| | | 3 | +++ | ++ | | | +++ | +++ | ++ | |
| | | 1.5 | +++ | ++ | | | +++ | +++ | ++ | |
| | | 0.75 | +++ | +++ | | | +++ | +++ | ++ | |
| | | 0.38 | ++ | +++ | | | +++ | +++ | ++ | |
| | | 0.18 | +++ | ++ | | | +++ | +++ | ++ | |
| | 0.5 | 6 | ++ | + | | | +++ | +++ | + | |
| | | 3 | ++ | + | | | +++ | +++ | + | |
| | | 1.5 | +++ | ++ | | | +++ | +++ | + | |
| | | 0.75 | ++ | ++ | | | ++ | +++ | ++ | |
| | | 0.38 | ++ | ++ | | | ++ | +++ | ++ | |
| | | 0.18 | ++ | ++ | | | ++ | +++ | ++ | |
| | 0.6 | 6 | ++ | | ± | + | ± | ++++ | 0 | 0 |
| | | 3 | +++ | | ++ | + | +++ | ++++ | + | + |
| | | 1.5 | ++++ | | +++ | +++ | ++++ | ++++ | +++ | ++ |
| | | 0.75 | +++ | | +++ | +++ | +++ | ++++ | +++ | ++ |
| | | 0.38 | +++ | | +++ | + | +++ | ++ | ++ | + |
| | | 0.18 | +++ | | +++ | ++ | +++ | ++ | + | + |
| | 0.7 | 6 | ++ | | ± | ± | +++ | ++++ | 0 | ± |
| | | 3 | + | | ± | ± | ++ | ++ | ± | ± |
| | | 1.5 | +++ | | ++ | + | +++ | +++ | ++ | ++ |
| | | 0.75 | +++ | | +++ | + | + | +++ | +++ | ++ |
| | | 0.38 | +++ | | +++ | +++ | +++ | + | +++ | +++ |
| | | 0.18 | +++ | | + | + | +++ | +++ | ++ | + |

TABLE 1. (continued)

| Buffer | Ionic strength | Protein coating (mg/ml) | Cell lysates | | | | Glucuronidase (mg/ml) | | Lysis buffer | Lysis buffer w/o lysozyme |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *E. coli* B | *E. coli* K12 | *E. coli* #222 and #329 | *Enterobacter* sp. | 0.1 | 0.01 | | |
| TPG | 0.8 | 6 | 0 | | 0 | 0 | + | ++ | 0 | |
| | | 3 | + | | ± | ± | ++ | +++ | ± | |
| | | 1.5 | + | | + | + | ++ | +++ | + | |
| | | 0.75 | ++ | | ++ | ++ | ++ | +++ | + | |
| | | 0.38 | ++ | | ++ | ++ | ++ | ++ | ++ | |
| | | 0.18 | ++ | | ++ | ++ | ++ | ++ | ++ | |
| | 1.0 | 6 | 0 | | 0 | 0 | + | ++ | 0 | |
| | | 3 | + | | ± | ± | ++ | +++ | 0 | |
| | | 1.5 | ++ | | + | + | ++ | +++ | + | |
| | | 0.75 | +++ | | +++ | ++ | +++ | +++ | ++ | |
| | | 0.38 | ++ | | ++ | ++ | ++ | ++ | ++ | |
| | | 0.18 | +++ | | +++ | +++ | ++ | +++ | +++ | |
| | 1.5 | 6 | 0 | | 0 | 0 | ± | ± | 0 | |
| | | 3 | 0 | | 0 | 0 | ± | + | 0 | |
| | | 1.5 | 0 | | 0 | 0 | + | ++ | 0 | |
| | | 0.75 | + | | + | + | + | ++ | + | |
| | | 0.38 | ++ | | ++ | ++ | ++ | ++ | ++ | |
| | | 0.18 | ++ | | ++ | ++ | + | ++ | ++ | |
| | 2.0 | 6 | 0 | | 0 | 0 | ± | ++ | 0 | |
| | | 3 | 0 | | 0 | 0 | + | ++ | 0 | |
| | | 1.5 | ± | | ± | ± | ++ | +++ | ± | |
| | | 0.75 | ± | | ± | ± | + | ++ | ± | |
| | | 0.38 | ++ | | ++ | ++ | + | +++ | ++ | |
| | | 0.18 | + | | + | + | ± | ++ | + | |

TABLE 1. (continued)

| Buffer | Ionic strength | Protein coating (mg/ml) | Cell lysates | | | | Glucuronidase (mg/ml) | | Lysis buffer | Lysis buffer w/o lysozyme |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *E. coli* B | *E. coli* K12 | *E. coli* #222 and #329 | *Enterobacter* sp. | 0.1 | 0.01 | | |
| Medcalf | 0.18 | 6 | ++ | ++ | | | +++ | +++ | + | |
| | | 3 | +++ | ++ | | | +++ | +++ | ++ | |
| | | 1.5 | ++ | ++ | | | +++ | +++ | + | |
| | | 0.75 | + | + | | | + | ++ | + | |
| | | 0.38 | + | + | | | + | ++ | + | |
| | | 0.18 | + | + | | | + | ++ | + | |
| | 0.35 | 6 | + | + | | | +++ | +++ | + | |
| | | 3 | ++ | ++ | | | ++ | +++ | + | |
| | | 1.5 | ++ | ++ | | | ++ | +++ | + | |
| | | 0.75 | ++ | ++ | | | ++ | ++ | + | |
| | | 0.38 | ++ | ++ | | | ++ | ++ | + | |
| | | 0.18 | ++ | ++ | | | ++ | ++ | + | |
| | 0.7 | 6 | ++ | | + | + | +++ | +++ | + | + |
| | | 3 | +++ | | ++ | + | + | ++ | ++ | + |
| | | 1.5 | ++ | | + | ++ | ++ | +++ | +++ | +++ |
| | | 0.75 | +++ | | +++ | ++ | +++ | +++ | ++ | + |
| | | 0.38 | ++ | | +++ | + | +++ | +++ | +++ | +++ |
| | | 0.18 | +++ | | +++ | ++ | +++ | +++ | +++ | + |
| | 1.4 | 6 | ++ | | ++ | + | ++ | +++ | + | |
| | | 3 | + | | + | + | + | ++ | + | |
| | | 1.5 | + | | + | + | + | + | + | |
| | | 0.75 | + | | + | + | + | + | + | |
| | | 0.38 | + | | ++ | + | + | ++ | + | |
| | | 0.18 | ++ | | + | + | ++ | + | + | |

TABLE 2. Effects of buffer types and saline concentrations on nonspecific agglutination of latex sensitized with four levels of each of four different anti-glucuronidase antibody preparations

| Buffer | Saline Concentration (M) | Molecular Probes (mg/ml protein applied) | | | |
|---|---|---|---|---|---|
| | | 0.07 | 0.06 | 0.05 | 0.04 |
| Glycine buffer (pH 8.2) | 0.43 | 0[a] | 0 | 0 | 0 |
| | 0.26 | 0 | 0 | 0 | 0 |
| | 0.17 | 0 | 0 | 0 | 0 |
| | 0.09 | 0 | 0 | 0 | 0 |
| | 0.04 | ± | 0 | 0 | 0 |
| | 0.02 | 0 | 0 | 0 | 0 |
| Borate buffer (pH 8.0) | 0.43 | ± | ± | 0 | 0 |
| | 0.26 | ± | ± | 0 | 0 |
| | 0.17 | ± | ± | 0 | 0 |
| | 0.09 | ± | ± | 0 | 0 |
| | 0.04 | ± | ± | 0 | 0 |
| | 0.02 | ± | ± | 0 | 0 |
| Tris/azide buffer (pH 7.2) | 0.43 | ± | ± | ± | ± |
| | 0.26 | ± | ± | ± | 0 |
| | 0.17 | ± | ± | 0 | 0 |
| | 0.09 | ± | 0 | 0 | 0 |
| | 0.04 | ± | 0 | 0 | 0 |
| | 0.02 | ± | 0 | 0 | 0 |
| Phosphate buffer (pH 6.6) | 0.43 | ± | ± | ± | ± |
| | 0.26 | ± | ± | ± | ± |
| | 0.17 | ± | ± | ± | ± |
| | 0.09 | ± | ± | ± | ± |
| | 0.04 | ± | ± | ± | ± |
| | 0.02 | ± | ± | ± | ± |

[a]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has some agglutinated particles on magnified examination; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed.

TABLE 2. (continued)

| Sample | Concentration | 5'3' (mg/ml protein applied) | | | |
|---|---|---|---|---|---|
| | | 0.07 | 0.06 | 0.05 | 0.04 |
| Glycine buffer (pH 8.2) | 0.43 | 0 | 0 | 0 | 0 |
| | 0.26 | 0 | 0 | 0 | 0 |
| | 0.17 | 0 | 0 | 0 | 0 |
| | 0.09 | 0 | 0 | 0 | 0 |
| | 0.04 | 0 | 0 | 0 | 0 |
| | 0.02 | 0 | 0 | 0 | 0 |
| Borate buffer (pH 8.0) | 0.43 | ± | ± | 0 | 0 |
| | 0.26 | ± | ± | 0 | 0 |
| | 0.17 | ± | ± | 0 | 0 |
| | 0.09 | ± | ± | 0 | 0 |
| | 0.04 | ± | ± | 0 | 0 |
| | 0.02 | ± | ± | 0 | 0 |
| Tris/azide buffer (pH 7.2) | 0.43 | 0 | ± | ± | 0 |
| | 0.26 | 0 | ± | 0 | 0 |
| | 0.17 | 0 | ± | 0 | 0 |
| | 0.09 | 0 | 0 | 0 | 0 |
| | 0.04 | 0 | 0 | 0 | 0 |
| | 0.02 | 0 | 0 | 0 | 0 |
| Phosphate buffer (pH 6.6) | 0.43 | ± | + | ± | ± |
| | 0.26 | ± | + | ± | ± |
| | 0.17 | ± | + | ± | ± |
| | 0.09 | ± | + | ± | ± |
| | 0.04 | ± | + | ± | ± |
| | 0.02 | ± | + | ± | ± |

TABLE 2. (continued)

| Sample | Concentration | Protein A (mg/ml protein applied) | | | |
|---|---|---|---|---|---|
| | | 0.07 | 0.06 | 0.05 | 0.04 |
| Glycine buffer (pH 8.2) | 0.43 | ± | ± | ± | 0 |
| | 0.26 | ± | ± | 0 | 0 |
| | 0.17 | 0 | 0 | 0 | 0 |
| | 0.09 | 0 | ± | ± | 0 |
| | 0.04 | 0 | 0 | 0 | 0 |
| | 0.02 | 0 | 0 | 0 | 0 |
| Borate buffer (pH 8.0) | 0.43 | 0 | 0 | 0 | 0 |
| | 0.26 | 0 | 0 | 0 | 0 |
| | 0.17 | 0 | 0 | 0 | 0 |
| | 0.09 | 0 | 0 | 0 | 0 |
| | 0.04 | 0 | 0 | 0 | 0 |
| | 0.02 | 0 | 0 | 0 | 0 |
| Tris/azide buffer (pH 7.2) | 0.43 | ± | ± | ± | 0 |
| | 0.26 | ± | ± | ± | ± |
| | 0.17 | ± | ± | ± | ± |
| | 0.09 | ± | ± | 0 | 0 |
| | 0.04 | ± | 0 | 0 | 0 |
| | 0.02 | ± | 0 | 0 | 0 |
| Phosphate buffer (pH 6.6) | 0.43 | ± | + | ± | ± |
| | 0.26 | ± | + | ± | ± |
| | 0.17 | ± | + | ± | ± |
| | 0.09 | ± | + | ± | ± |
| | 0.04 | ± | + | ± | ± |
| | 0.02 | ± | + | ± | ± |

TABLE 2. (continued)

| Sample | Concentration | Antigen-specific affinity chromatography (mg/ml protein applied) | | | |
|---|---|---|---|---|---|
| | | 0.07 | 0.06 | 0.05 | 0.04 |
| Glycine buffer (pH 8.2) | 0.43 | ± | ± | ± | 0 |
| | 0.26 | ± | ± | ± | 0 |
| | 0.17 | 0 | 0 | 0 | 0 |
| | 0.09 | 0 | 0 | 0 | 0 |
| | 0.04 | ± | ± | 0 | 0 |
| | 0.02 | 0 | 0 | 0 | 0 |
| Borate buffer (pH 8.0) | 0.43 | 0 | 0 | ± | 0 |
| | 0.26 | 0 | 0 | ± | 0 |
| | 0.17 | 0 | 0 | ± | 0 |
| | 0.09 | 0 | 0 | ± | 0 |
| | 0.04 | 0 | 0 | ± | 0 |
| | 0.02 | 0 | 0 | ± | 0 |
| Tris/azide buffer (pH 7.2) | 0.43 | ± | ± | ± | ± |
| | 0.26 | ± | ± | ± | ± |
| | 0.17 | ± | ± | ± | ± |
| | 0.09 | ± | ± | ± | 0 |
| | 0.04 | ± | ± | ± | 0 |
| | 0.02 | ± | ± | 0 | 0 |
| Phosphate buffer (pH 6.6) | 0.43 | ± | + | ± | + |
| | 0.26 | ± | + | ± | + |
| | 0.17 | ± | + | ± | + |
| | 0.09 | ± | + | ± | + |
| | 0.04 | ± | + | ± | + |
| | 0.02 | ± | + | ± | + |

TABLE 3. Effects buffer type and saline concentrations on nonspecific agglutination of latex sensitized with four different levels of protein A purified anti-glutamate decarboxylase antibody

| Buffer | NaCl Concentration (M) | Anti-glutamate decarboxylase latex (mg/ml protein applied) | | | |
|---|---|---|---|---|---|
| | | 0.1 | 0.08 | 0.06 | 0.04 |
| Glycine buffer (pH 8.2) | 0.43[a] | +[b] | ± | ± | 0 |
| | 0.26 | + | ± | 0 | 0 |
| | 0.17 | ± | 0 | 0 | 0 |
| | 0.086 | ± | 0 | 0 | 0 |
| | 0.043 | 0 | 0 | 0 | 0 |
| | 0.025 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| Borate buffer (pH 8.0) | 0.43 | ± | 0 | 0 | 0 |
| | 0.26 | ± | 0 | 0 | 0 |
| | 0.17 | ± | 0 | 0 | 0 |
| | 0.086 | 0 | 0 | 0 | 0 |
| | 0.043 | 0 | 0 | 0 | 0 |
| | 0.025 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| Tris buffer (pH 6.0) | 0.43 | ± | 0 | 0 | 0 |
| | 0.26 | ± | 0 | 0 | 0 |
| | 0.17 | ± | 0 | 0 | 0 |
| | 0.086 | ± | 0 | 0 | 0 |
| | 0.043 | ± | 0 | 0 | 0 |
| | 0.025 | ± | 0 | 0 | 0 |
| | 0 | ± | 0 | 0 | 0 |
| Phosphate buffer (pH 6.6) | 0.43 | ++ | ± | ± | 0 |
| | 0.26 | ++ | ± | ± | 0 |
| | 0.17 | ++ | ± | ± | 0 |
| | 0.086 | ++ | ± | ± | 0 |
| | 0.043 | ++ | ± | ± | 0 |
| | 0.025 | ++ | ± | ± | 0 |
| | 0 | ++ | ± | ± | 0 |
| Phosphate buffer (pH 8.1) | 0.43 | 0 | 0 | 0 | 0 |
| | 0.26 | 0 | 0 | 0 | 0 |
| | 0.17 | 0 | 0 | 0 | 0 |
| | 0.086 | 0 | 0 | 0 | 0 |
| | 0.043 | 0 | 0 | 0 | 0 |
| | 0.025 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |

[a]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has some agglutinated particles on magnified examination; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed; ++ reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles.

TABLE 4. Latex agglutination reactions obtained when four different blocking agents were tested with 0.8 and 0.2 μm diameter latex sensitized with anti-glutamate decarboxylase

| Latex | Blocking treatment[a] | Cell lysates[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *E. coli* C | *E. coli* B | *E. coli* NOS | *E. coli* 329 | *E. coli* 222 | *Klebsiella* sp. | *Enterobacter* sp. | Lysis buffer |
| Anti-glutamate decarboxylase - 0.8 μm latex | co-blocked | + + | + + | + + | + + | + + | + | + | + |
| | casein-thimerosal | + + | + + | + + | + + | + + | + | + | + |
| | casein-BSA | + + + | + + | + | + + | + + | + | + | + |
| | casein-glycerol | + + + | + + | + + + | + + | + + + | + | + | + |
| Anti-glutamate decarboxylase - 0.2 μm latex | coblocked | + | + | + | 0 | + + | 0 | 0 | 0 |
| | casein-thimerosal | + + | + + | + | + | + + + | 0 | ± | 0 |
| | casein-BSA | + + + | + + + + | + + | + | + + + + | 0 | 0 | 0 |
| | casein-glycerol | + + + | + + + | + + | ± | + + + | 0 | 0 | 0 |

[a]Coblocked = for 0.2 μm latex - 2.25 mg/ml antibody + 0.075 mg/ml casein and for 0.8 μm latex - 0.5 mg/ml antibody + 0.02 mg/ml casein; casein-thimerosal = included 0.02% (w/v) thimerosal; casein-BSA = 0.33 mg/ml casein + 0.165 mg/ml bovine serum albumin; casein-glycerol = 5 mg/ml casein + 5% glycerol.

[b]Numbers and letters refer to strain used (for reference only). Cultures were not all at the same growth stage; cultures were grown only for 24 h and differences among the species and strains were not adjusted for.

[a]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has some agglutinated particles on magnified examination; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed; + + reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles; + + + reaction has several agglutinated particles of a moderate size with a relatively clear background; + + + + reaction has one or more agglutinated particle clumps with no macroscopically free particles.

TABLE 5. Effects of sodium salicylate on agglutination results obtained with 0.2 μm latex sensitized with four different concentrations of anti-tryptophanase or anti-glutamate decarboxylase.

| Latex | Sodium salicylate concentration | Antibody concentration (mg/ml) | Cell lysates | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *E. coli* B[a] | *E. coli* C | *E. coli* 222 | *E. coli* 329 | *Enterobacter* sp. | *Klebsiella* sp. | *Citrobacter* sp. | *Proteus* sp. |
| Anti-tryptophanase 0.2 μm | 0 | 6 | ++[b] | +++ | +++ | +++ | +++ | +++ | ++ | +++ |
| | | 3 | ++++ | +++ | +++ | +++ | +++ | +++ | ++ | +++ |
| | | 1.5 | + | +++ | ++ | ++ | + | +++ | ++ | 0 |
| | | 0.5 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |
| Anti-tryptophanase 0.2 μm | 100 mM | 6 | + | + | ++ | + | 0 | + | ± | 0 |
| | | 3 | + | ± | +++ | + | 0 | 0 | 0 | 0 |
| | | 1.5 | 0 | 0 | ++ | 0 | 0 | 0 | 0 | 0 |
| | | 0.5 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 |
| Anti-glutamate decarboxylase 0.2 μm | 0 | 6 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | | 3 | +++ | ++ | +++ | ++ | +++ | +++ | +++ | ++ |
| | | 1.5 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ |
| | | 0.5 | +++ | +++ | +++ | ++ | + | + | + | 0 |
| Anti-glutamate decarboxylase 0.2 μm | 100 mM | 6 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | 0 |
| | | 3 | ++ | +++ | +++ | ++ | ++ | ++ | ++ | 0 |
| | | 1.5 | +++ | +++ | +++ | +++ | ++ | +++ | ++ | 0 |
| | | 0.5 | + | ++ | +++ | ++ | 0 | 0 | + | 0 |

[a]Numbers and letters refer to strain used (for reference only). Cultures were not all at the same growth stage; cultures were grown only for 24 h and differences among the species and strains were not adjusted for.

[b]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has some agglutinated particles on magnified examination; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed; ++ reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles; +++ reaction has several agglutinated particles of a moderate size with a relatively clear background; ++++ reaction has one or more agglutinated particle clumps with no macroscopically free particles.

# PART 5. LATEX AGGLUTINATION TESTS WITH CELL CULTURE LYSATES AND COMMERCIAL ENZYMES

## INTRODUCTION

Results obtained when 0.2-$\mu$m diameter sensitized latex particles were blocked with ß-casein and glycerol (Part 3 of this dissertation) indicated that the assays had the potential to distinguish among positive and negative control organisms. To further evaluate the assays, the full sensitivity and specificity of the reagents needed to be determined. Results reported in Part 1 (of this dissertation) demonstrated that antibodies elicited to one *E. coli* enzyme might cross-react with antibodies elicited to a different *E. coli* enzyme. Therefore, in a practical situation the latex agglutination tests might not fully distinguish among strains of *E. coli* that were negative for a specific trait (tryptophanase, glutamate decarboxylase, or glucuronidase). A moderate amount of nonspecific agglutination also was still present, but it was not known if it was sufficient to cause substantial interference. Only by testing of the latex preparations with actual cell culture lysates could valid conclusions about the usefulness of the latex agglutination assays be made.

The original project idea was to develop a latex agglutination assay that could detect the presence of three distinct bacterial enzymes on the basis of colored latex particles separating as a result of one or more agglutination reactions. Results of previous tests, however, did not indicate that the assay was sufficiently developed for combined testing. Results from the use of sodium salicylate established that different parameters were necessary for each individual assay. Each assay was therefore tested on the basis of individually optimized reaction conditions. The optimized conditions were determined by testing cell lysates of six control organisms (three positive and three negative) and adjusting reaction parameters accordingly. The optimum reaction conditions were then applied to this larger study of known cultures to determine specificity and sensitivity.

## MATERIALS AND METHODS

### Preparation of Sensitized Latex

Three separate batches of sensitized anti-enzyme latex were made. From a pooled batch of antigen-specific affinity chromatography purified antibody preparations, a portion of each antibody preparation was adjusted to approximately 1 and 0.5 mg/ml protein by using $A_{280}$ values and an extinction coefficient of 1.4 (1, 3). Two milliliters of each antibody preparation, were added to three 12 × 75 mm test tubes and prewarmed to 37°C. Ten milligrams of dialysis cleaned dyed 0.22 μm diameter latex were added to each test tube. Blue dyed latex was used for anti-glutamate decarboxylase, red dyed latex was used for anti-tryptophanase, and yellow dyed latex was used for anti-glucuronidase. The tubes were covered with Parafilm (American National Can, Greenwich, CT) and mixed by inversion several times. The latex suspensions were incubated at 37°C for a minimum of 2 h. During the 2 h incubation, they were mixed by inversion approximately every 15 min for the first hour and left stationary the second hour. After incubation, 0.25 mg of latex was removed to determine the quantity of latex bound protein (see Latex Bound Protein Determinations). One milliliter of blocking solution (10 mg/ml ß-casein [Sigma, product number C-6905], 10% glycerol [Fisher Scientific, product number G33-500], in Tris/azide buffer [0.03 M, 0.1% BSA, 0.05% sodium azide, pH 7.4]) was added and the latex suspensions were incubated overnight at 4°C. The following day, using a Sorval model RT6000B centrifuge (Sorval Instruments, DuPont Co., Newton CT), the latex was sedimented at 3400 RPM (2410 × *g*) until a latex pellet formed and the supernate was clear. This was accomplished in approximately 3 h. All except approximately 0.5 ml, the supernate was removed. The latex pellets were each resuspended in the remaining supernates and the latex suspensions were transferred to 2.0 ml microcentrifuge tubes. The volume of each tube was increased to approximately 1.5 ml with filtered blocking solution. The latex was washed three times by centrifugation washing using assay buffer (0.03 M Tris, 5 mg/ml

BSA, 0.02 mg/ml ß-casein, 5% glycerol, and 0.05% sodium azide, pH 7.4) as the wash buffer. The latex was resuspended to a concentration of 1% (w/v) latex in assay buffer and stored at 4°C.

Each batch of latex was tested against cell lysates to determine reactivity. Three phenotypically negative (non-*E. coli*) and three phenotypically positive (*E. coli*) organisms were used to prepare the cell lysates. The cell lysates were used to determine the optimal level of sodium salicylate that should be added, depending on the enzyme that was to be detected (data not shown). The optimum sodium salicylate concentration was the concentration at which non-*E. coli* cell lysates did not result in agglutination whereas the *E. coli* cell lysates did produce agglutination reactions. Later, pooled batches of latex were tested by using the revised assay parameters.

**Testing of the Latex Reagents**

The latex suspensions were tested by using enzyme preparations and cell lysates. Cell lysates, from known cultures grown in EIB for 24 h, were prepared as previously described. When commercial enzyme preparations were used, they were prepared in 1 ml (total volume) of cell lysis buffer (see Cell Lysis previously described) and further diluted in the lysis buffer to the appropriate concentrations. In addition, preparations of tryptophanase and glutamate decarboxylase, from Dr. David Metzler, were diluted 0.1 to 0.000001 mg of enzyme per ml of lysis buffer.

An unanticipated problem arose during these studies. When the RNAse reagent used in the lysis procedure was exhausted and replaced with fresh reagent, the pattern of nonspecific agglutination reactions suddenly changed. Because of these differences in the RNAse reagent, further adjustments in the concentrations of sodium salicylate (or polyethylene glycol) had to be made to achieve the appropriate agglutination reactions for the control organisms.

**Calculation of Sensitivity, Specificity, and Efficiency of the Latex Agglutination Assays**

Sensitivity and specificity were calculated on the basis of the results of cell lysate tests. The formulas for the calculation of sensitivity and specificity (2, 5) are:

$$\text{SENSITIVITY} = \frac{\text{TRUE POSITIVES}}{\text{TRUE POSITIVES} + \text{FALSE NEGATIVES}} \times 100\%$$

$$\text{SPECIFICITY} = \frac{\text{TRUE NEGATIVES}}{\text{TRUE NEGATIVES} + \text{FALSE POSITIVES}} \times 100\%$$

Determinations of true and false reactions were made by comparing the phenotypic traits found by conventional methods with the results of the latex agglutination tests. If a culture was phenotypically positive and agglutination did not occur for that enzyme, this was considered a false-negative reaction. Conversely, if a culture was phenotypically negative but agglutination occurred in the presence of the cell lysate, this was considered a false-positive reaction.

## RESULTS AND DISCUSSION

### Culture Lysate Results

Table 1 summarizes the results obtained when different batches of sensitized latex were prepared on different days by using the same protocol and antigen-specific affinity purified antibodies from the same purification pool. The results obtained with different batches of latex differed. Because of this, different batches of sensitized latex were mixed for some tests. The results of using mixed latex reagents to test culture cell lysates are summarized in Table 2. Mixing the latex batches provided sufficient quantities of uniform preparations of sensitized latex for more extensive cell-lysate testing.

The sensitivities and specificities of the assays were determined from the results summarized in Tables 1 and 2. The average sensitivity of anti-tryptophanase was 77% with a specificity of 61%; these were insufficient to be a useful assay procedure. Anti-glutamate decarboxylase sensitized latex had a sensitivity of 83% and a specificity of 57%. Anti-glucuronidase sensitized latex had a sensitivity of 70% and a specificity of 82%. Some negative reactions occurred with *E. coli* strains, but these were generally consistent with the phenotypic characterizations. Most of the positive reactions with non-*E. coli* were inconsistent with the phenotypic characterizations.

There are two general explanations for the inadequate performance of these assays. First, nonspecific agglutination reactions were not sufficiently eliminated from the assays. Second, cross-reactions (positive agglutination) were obtained with species other than *E. coli*. The initial problem encountered in the development of the assay was nonspecific agglutination (Part 2 of this dissertation). Although these nonspecific agglutination problems were reduced (Part 3 of this dissertation), they were not completely eliminated (Tables 1 and 2). Furthermore, tests on cell lysates showed that an altogether higher degree of non-specificity existed in agglutination reactions

obtained when non-*E. coli Enterobacteriaceae* were tested.

**Results With Commercial Enzymes**

To test the latex preparations for specificity and cross-reactivity, with commercial enzymes themselves, enzyme preparations in lysis buffer were used as antigen sources. The approximate sensitivities of the latex suspensions (Table 3) were: 10 μg/ml (0.01 mg/ml) for anti-tryptophanase, 0.1 μg/ml (0.0001 mg/ml) for anti-glutamate decarboxylase, and 1 μg/ml (0.001 mg/ml) for anti-glucuronidase. These results demonstrate that the sensitized latex preparations ranged from sensitive (anti-tryptophanase) to highly sensitive (glutamate decarboxylase).

Anti-glucuronidase sensitized latex reacted with as little as 1 μg/ml of glucuronidase (Table 3). Considering that this latex failed to readily react with cell lysates leads to the belief that cell lysates yielded insufficient free glucuronidase for a readily detectable latex agglutination reaction. This occurred despite the positive reactions seen when the cultures were grown in EIB-MUG medium. Kaspar (4) encountered a similar problem in his coagglutination assay for glucuronidase. Kaspar found that his coagglutination anti-glucuronidase assay required a minimum of 31 μg/ml of glucuronidase for detection; 500 μg/ml was optimal. According to Kaspar (4), the anti-glucuronidase coagglutination assay was the least sensitive and most difficult of all the coagglutination reactions to visualize. Although these studies disclosed the sensitivity of the anti-glucuronidase latex agglutination assay to be about 1 μg/ml (Table 3), this was insufficient for adequate detection of the enzyme in cell lysates. A procedural difference, however, between Kaspar's coagglutination assay and the latex agglutination assay described here should be mentioned. The coagglutination reaction was observed with the aid of magnification. In the latex agglutination reaction, differentiation at this level was often not conclusive. The ± reactions (those observed only with the aid of magnification) were sometimes background level because of

nonspecific agglutination. Therefore, stronger, macroscopic agglutination, was the goal of these latex agglutination assays.

The sensitivities of the other latex agglutination assays also were compared to the sensitivities of the anti-enzyme coagglutination assay developed by Kaspar (4). Kaspar's anti-tryptophanase coagglutination assay (4) could detect 2-3 $\mu$g/ml of commercial enzyme preparation but was unable to detect any enzyme in cell lysates. Although the latex assay was evidently unable to achieve the sensitivity Kaspar claimed for his coagglutination assay, it did detect positive reactions with cell lysates. Because of cross-reactions with other commercial enzymes (reported later), despite antigen-specific affinity chromatography purification of the antibody preparations, determinations of which substances were acting as the antigens can only be speculated. The latex assay for glutamate decarboxylase was about an order of magnitude more sensitive than Kaspar's coagglutination assay (4); 0.1 $\mu$g/ml for the latex assay versus 2-3 $\mu$g/ml for the coagglutination assay.

Cross-reactivities among commercial enzyme preparations and latex suspensions are shown in Table 4. All latex suspensions cross-reacted with 100 $\mu$g/ml (0.1 mg/ml) of the other enzymes, and sometimes cross-reactivity developed at even lower enzyme concentrations. All latex preparations reacted readily with 1 mg/ml of enzyme preparation (data not shown). Only at 0.1 mg/ml were differences in reactivities evident.

Anti-tryptophanase sensitized latex gave a very strong agglutination reaction with the commercial glutamate decarboxylase enzyme preparation; the agglutination reaction with 10 $\mu$g of glutamate decarboxylase per ml was as strong or stronger than with 10 $\mu$g of tryptophanase per ml. The anti-tryptophanase sensitized latex also reacted with 0.1 mg/ml of glucuronidase. As expected, this was consistent with the reactivities of the anti-tryptophanase in the Western blot studies (Part 1 of this dissertation). The Ouchterlony immunodiffusion results (Part 1 of this dissertation), however,

did not suggest that antigen-specific affinity chromatography purified antibody preparations would cross-react with the other enzyme preparations.

Anti-glutamate decarboxylase-sensitized latex also had cross-reactions to the other enzymes (Table 4). Cross reactivity with both tryptophanase and glucuronidase resulted at concentrations of 0.1 mg enzyme per ml of lysis buffer. Again, as expected, the results were consistent with the Western blot studies (Part 1 of this dissertation). The results (Table 4) were consistent with some of the Ouchterlony immunodiffusion results (Part 1 of this dissertation). Antigen-specific affinity chromatography purified antibody preparations indicated that cross-reactivity occurred with tryptophanase. Anti-glucuronidase cross-reactivity, however, was not detected.

Anti-glucuronidase sensitized latex exhibited the most severe cross-reactivities with the other enzymes (Table 4). As stated before, the anti-glucuronidase-sensitized latex preparation did not consistently detect glucuronidase activity in cell lysates, but latex preparation could detect approximately 1 $\mu$g of glucuronidase per ml of lysis buffer tryptophanase (Table 3).

The use of different tryptophanase and glutamate decarboxylase preparations (from Dr. David Metzler) did not significantly change the cross-reactivity patterns detected (data not shown). Significant cross-reactivities were still present when the different glutamate decarboxylase and tryptophanase preparations were used as antigens. The cross-reactivities were probably a result of the heterogeneous polyclonal antibody preparations used to sensitize the latex.

There are three possible explanations for the cross-reactivity of the assays: the presence of enzymes in species not normally considered having the enzymes, cross-reactivity with similar structural proteins, or nonspecific antibody preparations. Based on the present studies and the biochemical phenotypes of the bacterial cultures used, the latter explanation is most likely.

## SUMMARY AND CONCLUSIONS

Cell lysates of members of the *Enterobacteriaceae* were used for preliminary evaluations of the anti-enzyme latex agglutination preparations. These tests were conducted to determine if the assays would be satisfactory for use by industrial and governmental food and water analysts. Evaluations of the assays in circumstances under which they would be employed (Tables 1 and 2) were necessary to decide if the assays were specific and sensitive enough to replace or supplement current methods. Although the assays were sensitive (Table 3), they were not sufficiently specific (Tables 1, 2, and 4). It has been noted previously (2) that as the sensitivity of immunoassays increase, there is often a decrease in specificity. Tests on cell lysates from additional cultures could have been made. Results of the tests were not good enough, however, to warrant further work with the current assays.

The anti-enzyme latex agglutination assays suffered from heterologous antibody preparations which caused nonspecific agglutination reactions. The full extent of the problems with the antibody preparations, however, was not realized until the assays were used in the evaluation of the cell lysates. Although there were early indications that cross-reactions might pose an obstacle to the assay (Part 1 of this dissertation), problems with nonspecific agglutination reactions were dealt with first (Parts 2 and 3 of this dissertation). Nevertheless, anti-enzyme agglutination tests do appear feasible. Latex agglutination assays are easier to interpret than coagglutination assays, and multiple indicators (tests for different enzymes) could be combined in a multianalyte assay. Like coagglutination assays, latex assays are rapid and simple to use. It is clear that additional work is required to obtain better antibody preparations. Whether or not different antibody preparations would solve all of the problems encountered in this study, however, remains unresolved.

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TABLE 1. Number of positive latex agglutination tests using cell lysates

| Species | Number of strains tested | Latex agglutination test[bc] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Anti-Tryp[a] (8/92) | Anti-Tryp (9/92) | Anti-GAD (8/92) | Anti-GAD (9/92) | Anti-GUD (8/92) | Anti-GUD (9/92) |
| *E. coli* | 22 | 22 | 21 | 18 | 16 | 22 | 16 |
| *Citrobacter spp.* | 2 | 0 | 0 | 0 | 1 | 0 | 0 |
| *Klebsiella spp.* | 2 | 1 | 1 | 0 | 1 | 1 | 0 |
| *Enterobacter spp.* | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Hafnia spp.* | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Proteus spp.* | 3 | 2 | 0 | 0 | 0 | 0 | 0 |
| *Shigella spp.* | 2 | 2 | 1 | 0 | 0 | 1 | 1 |
| *Salmonella spp.* | 2 | 1 | 1 | 0 | 0 | 2 | 1 |

[a]Tryp = tryptophanase; GUD = glucuronidase; GAD = glutamate decarboxylase.
[b]Numbers refer to date of latex batch preparation; for reference only.
[c]Sodium salicylate (50 mM) was added to latex buffer to control nonspecific agglutination.

TABLE 2. Number of positive latex agglutination results using cell lysates with a different batch of sensitized latex

| Species | Number of strains tested | Latex agglutination test | | | | | |
|---|---|---|---|---|---|---|---|
| | | Anti-Tryp[a] 200 mM[b] | Anti-Tryp 250 mM | Anti-GAD 200 mM | Anti-GAD 250 mM | Anti-GUD 200 mM | Anti-GUD 50 mM |
| *E. coli* | 8 | 7 | 5 | 7 | 6 | 0 | 0 |
| *Citrobacter spp.* | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| *Klebsiella spp.* | 4 | 3 | 2 | 1 | 0 | 0 | 0 |
| *Enterobacter spp.* | 4 | 3 | 1 | 2 | 0 | 0 | 0 |

[a]Tryp = tryptophanase; GUD = glucuronidase; GAD = glutamate decarboxylase.
[b]The concentration of sodium salicylate included in the assay buffer.

TABLE 3. Sensitivity of latex with commercial enzyme preparations

| Latex | Assay conditions[a] | Appropriate[b] enzyme preparations in lysis buffer (mg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.1 | 0.01 | 0.001 | 0.0001 | 0.00001 | 0.000001 |
| Anti-tryptophanase | 100 mM SS | ++++ | ± | 0 | 0 | 0 | 0 |
| Anti-glutamate decarboxylase | 225 mM SS | ++++ | +++ | + | ± | 0 | 0 |
| Anti-glucuronidase | 4% PEG | +++ | ++++ | ± | 0 | 0 | 0 |

[a]The amount of sodium salicylate (SS) or polyethylene glycol (PEG) used to stabilize the sensitized latex.
[b]Appropriate enzyme refers to using the enzyme against which the latex was sensitized with the anti-enzyme antibodies (tryptophanase, glutamate decarboxylase, or glucuronidase).

TABLE 4. Cross-reactivity of latex with commercial enzyme preparations

| Latex | Assay conditions[a] | Enzyme preparation used[b] | Concentration of enzyme preparations in lysis buffer (mg/ml) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0.1 | 0.01 | 0.001 | 0.0001 | 0.00001 | 0.000001 |
| Anti-Tryptophanase | 0 mM SS | Tryp | ++++ | ± | 0 | 0 | 0 | 0 |
| | | GAD | +++ | + | 0 | 0 | 0 | 0 |
| | | GUD | + | 0 | 0 | 0 | 0 | 0 |
| Anti-glutamate decarboxylase | 200 mM SS | Tryp | + | ± | 0 | 0 | 0 | 0 |
| | | GAD | ++++ | ++ | + | ± | 0 | 0 |
| | | GUD | + | 0 | 0 | 0 | 0 | 0 |
| Anti-glucuronidase | 4% PEG | Tryp | +++ | ++ | ++ | ++ | ++ | ++ |
| | | GAD | ++ | ++ | ++ | ++ | ++ | ++ |
| | | GUD | +++ | ++++ | ++ | 0 | 0 | 0 |

[a]The amount of sodium salicylate (SS) or polyethylene glycol (PEG) used to stabilize the sensitized latex.
[b]Tryp = tryptophanase; GAD = glutamate decarboxylase; GUD = glucuronidase.
[c]Key to reactions: 0 reaction contains no agglutinated particles; ± reaction appears negative macroscopically but has some agglutinated particles under magnification; + reaction has a grainy appearance macroscopically on the basis of a few agglutinates formed; ++ reaction has discernible agglutinated clumps on a field of individual or slightly agglutinated particles; +++ reaction has several agglutinated particles of a moderate size with a relatively clear background; ++++ reaction has one or more agglutinated particle clumps with no macroscopically free particles.

## GENERAL SUMMARY AND CONCLUSIONS

The development of rapid and sensitive methods to detect *E. coli* in food and water, after a brief period of enrichment, would tremendously enhance the safety of perishable foods and potable water and would further protect consumers from the potential of food and waterborne illnesses. The original intent of the project was to develop a rapid, 10-minute assay that could eliminate the need for time-consuming cultural confirmation of initially presumptive cultures to establish the presence of *E. coli*.

Antibody preparations were developed against three selected *E. coli* enzymes: glutamate decarboxylase, glucuronidase, and tryptophanase. These enzymes were chosen because, taken together, they would provide a highly sensitive and specific indication of the presence of any *E. coli*, including verotoxin-producing strains.

Sensitivity and specificity are the most important parameters of immunoassays (1, 6, 11). Although antibodies were prepared by using procedures that were apparently successful in previous studies (8, 10), the antibody preparations were too heterogeneous to be used in latex agglutination assays. They reacted with proteins in the antigen preparations and cell lysates that were not the intended target antigens. Although this problem was recognized, its severity was not fully realized until late in the study, otherwise, more specific antibody preparations would have been prepared from more highly purified enzyme preparations or monoclonal antibodies could have been attempted. The concurrent presence of other problems with the assays directed attention to other studies in the belief that many of the problems with autoagglutination and cross-reactions might be resolved by appropriate conditions for latex preparation and use.

Evaluation of immunoglobulin binding to the latex particles provided a starting point for the investigation of causes of nonspecific agglutination reactions. Nonspecific agglutination reactions were reduced but not completely eliminated by the selection of several methods used for

contending with them. These included latex particle size, sensitization procedures, alternative blocking agents, type and pH of buffer and changes in the ionic conditions of the assay. Two methods were discovered that substantially reduced the number and intensity of the nonspecific agglutination reactions: the use of 0.22-$\mu$m diameter latex particles and use of a mixture of ß-casein and glycerol as the blocking reagents. Reductions in nonspecific agglutination reactions permitted better evaluation of the assays developed.

The latex agglutination assays, when tested against cell lysates from known cultures, failed to achieve useful levels of sensitivity and specificity. Sensitivity and specificity of the assays suffered from cross-reactions among the antibody preparations and the intended target antigens. It was concluded that the cross-reactivities were probably the result of the heterogeneity of the antibody preparations.

As stated previously, nonspecific agglutination of sensitized latex was a major problem in development of the *E. coli* enzyme latex agglutination tests. The greatest problem, however, was contending with cross-reactions. The results suggest that more highly purified enzyme preparations need to be used for immunization to produce polyclonal antibodies of suitable homogeneity for use in latex agglutination tests. Even more specific antibodies than polyclonals may be required, however, for the assay to become practical. The development of monoclonal antibodies to tryptophanase, glutamate decarboxylase, and/or glucuronidase (or other enzymes unique to *E. coli*) might be necessary to completely eliminate cross-reactions and achieve a satisfactory degree of specificity for these assays. Once the problems of specificity and cross-reactivity are solved, the assays should be sensitive enough to detect $10^5$ to $10^6$ *E. coli* per milliliter or per gram of sample.

In conclusion, the present work establishes the groundwork for the development of an anti-enzyme latex agglutination assay to detect *E. coli*. This is the first time that an anti-enzyme latex

agglutination test has been described. Some of the methods necessary for development of this assay are presented. More specific antibody preparations, however, are needed.

Several alternative formats that might utilize specific antibodies to selected *E. coli* enzymes also are possible. Rapid filter-based particle assays, latex chromatography, or magnetic particles are several of the methods that could be exploited to aid in the rapid confirmation of *E. coli* from presumptive cultures.

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